Design and Synthesis of Substrates and Inhibitors of α-methylacyl-CoA racemase

Submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor of Philosophy

UNIVERSITY OF LIVERPOOL

By

Ralph Kirk

Contents

Acknowledgements

First and foremost, my utmost gratitude to Dr Andrew Carnell, not only for his enthusiasm and intellectual input on this project but whose patience, friendliness and encouragement I will not forget. I would like to thank Dr Rob Gibson and Professor Chien-Feng for their contributions in this work. I would also like to thank Rick Cosstick for being actively interested in the project and for his contributions in our group meetings.

I extend my thanks to the past and present members of the Carnell and Cosstick groups, all of whom have made the lab a more interesting place to work – whether it being the occasional tea, the eclectic music, or the (in)frequent sulfur odor emanating from some corner of the lab. I'd like to thank Inder and Matt for reading (and re-reading) this work and for listening to my endless twitterings. In particular, I'd like to mention Dr James Gaynor, whose support, friendship and advice over the last few years have been invaluable.

On a more personal note, I would like to mention Chris, John, Paul, Rich and Rob (in alphabetical order) for their encouragement, welcomed distraction from work and the occasional drink.

On another personal note, I'd like to thank Emma for her love and support and for putting up with me thick and thin. You've done a good job at keeping my life interesting over the last few years.

I'd also like to thank Lee and Ben, for always being 'big brothers,' for knowing that they're always there if needs be. I hope they know it's reciprocated.

Finally, I am eternally grateful to Mum and Dad. Throughout my life they have been two pillars of unwavering love and support. Without them, this thesis would have been nothing but a pipedream.

Abstract

Around 30,000 men in the UK are diagnosed with prostate cancer (PCa) each year. The enzyme α-methylacyl-CoA racemase (AMACR) has been found to be overexpressed in prostate, colon, and other cancers. More recently, siRNA knockdown of the AMACR gene expression has been shown to inhibit proliferation of the cancerous prostate cells and therefore partially validate AMACR as a potential therapeutic target for the disease.[1] AMACR has a wide substrate specificity; its natural role is to catalyse the interconversion of (*R*) and (*S*)-stereoisomers at the α-position of branched chain acyl-CoA esters such as pristanoyl and trihydroxycholestanoyl-CoA. This provides the required (*S*)-configured substrate for the stereospecific oxidase enzyme for β-oxidation. It is also involved in the metabolism of Ibuprofen.

Recent X-ray crystallography studies on *Mycobacterium tuberculosis* α-methylacyl-CoA racemase (MCR) have led to a proposed mechanism for its catalytic activity. Kinetic and structural data suggest that a 1,1-proton transfer takes place, catalysed by the two active site bases His-126 and Asp-156, resulting in formation of an enzyme stabilised enolate.

Our group has recently synthesised a range of competitive inhibitors which are fluorinated analogues of the natural substrates. The most potent of these is 2-trifluoromethyltetradecanoyl-CoA (*Ki* = 1μM) that may work by lowering the p*K*a of the α-hydrogen and promoting enolisation. We have improved the synthesis of this compound and have prepared a new range of potential inhibitors. It is understood that the coenzyme A thioester of Ibuprofen has a better binding affinity towards AMACR than its natural substrates and also acts as a mild competitive inhibitor. This inspired us to synthesise Ibuprofen derivatives which when thioesterfied may exhibit better inhibition characteristics. β–Trifluoroibuprofen has shown some promising inhibition characteristics as a prodrug.

We have also designed three different assays (HPLC, LC-MS and chromogenic) in order to test the efficacy of our new series of inhibitors. Quantification of extent of racemisation by monitoring deuterium exchange (LC-MS) is our most efficient assay to date.

Abbreviations

34βE12	Keratin 903
Å	Angstrom
Ac	Acetyl
ACP	Acyl carrier protein
ACAA1	acetyl-CoA acyltransferase 1
ACOX	Peroxisomal acyl-coenzyme A oxidase
AMACR	α-Methylacyl-CoA racemase
AR	Androgen receptor
Arg	Arginine
Asp	Aspartate
AU	Absorbance units
Baif	CoA-hydrolase
Bcl-2	B-cell lymphoma 2
Bn	Benzyl
BPH	Benign prostatic hyperplasia
Bu	Butyl
C	Centigrade
ca.	Circa
CaiB	L-carnitine dehydratase
CDI	Carbonyldiimidazole
cDNA	complementary DNA

Chiral AGP	Chiral column packed with α-1 acid glycoprotein
Chiral OJ	Chiral column packed with Cellulose tris (4-methylbenzoate) coated on 10μm silica-gel
CI	Chemical ionisation
cm^{-1}	Wavenumber
CoA	Coenzyme A
COX	Cyclooxygenase
Cys	Cysteine
d	Doublet
D_2O	Deuterated water
Da	Dalton
DAST	Diethylamino sulphur trifluoride
DBP	D-specific bifunctional protein
DCC	*N, N'*-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMPU	1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone
DMF	*N, N'*-dimethylformamide
DMSO	Dimethylsulfoxide
E1cB	E-elimination, 1cb-first order with respect to conjugate base

e.e	Enantiomeric excess
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
eq	Equivalent
ES	Electrospray
Et	Ethyl
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
FAS	Fatty acid synthase
FACL	Fatty acid-CoA ligase
FAD	Flavin adenine dinucleotide
FRC	Formyl-CoA transferase
FT-IR	Fourier transform-infrared
GC	Gas chromatography
Glu	Glutamate
GSK	GlaxoSmithKline
h	Hours
His	Histidine
HOBt	1-Hydroxybenzotriazole
HPC	Hereditary Prostate Cancer
HPLC	High performance liquid chromatography
hr	Hour

HRMS	High resolution mass spectrometry
Hz	Hertz
Ile	Isoleucine
IPA	Isopropyl alcohol
*i*Pr	Isopropyl
J	Coupling constant (Hz)
*K*i	Inhibition constant
*K*m	Michaelis constant
KPi	Potassium phosphate
LBP	L-specific bifunctional protein
LC	Liquid chromatography
LDA	Lithium diisopropylamide
Leu	Leucine
LWB	Lineweaver-Burk
Lys	Lysine
m	Multiplet
m/z	Mass to charge ratio
MCR	AMACR homologue from *M. tuberculosis*
Me	Methyl
Met	Methionine
MFE	Multifunctional enzyme
mg	Milligrams

min	Minutes
ml	Millilitres
mmol	Millimoles
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
NAC	*N*-acetylcysteamine
NBS	*N*-bromosuccinimide
NCS	*N*-chlorosuccinimide
ng	nanogram
nm	nanometre
NMR	Nuclear magnetic resonance spectroscopy
NMP	*N*-Methyl-2-pyrrolidone
O.N	Over night
p53	Tumor protein 53
p63	Tumor protein 63
PCa	Prostate cancer
PCC	Pyridinium chlorochromate
PCR	Polymerase chain reaction
pKa	acid dissociation constant
Ph	Phenyl
PLP	Pyridoxyl phosphate
ppm	Parts per million

ppt	Precipitate
Pro	Proline
PSA	Prostate specific antigen
q	Quartet
RP-HPLC	Reverse phase HPLC
rt	Room temperature
s	Singlet or second
S_N1	Unimolecular nucleophilic substitution
S_N2	Bimolecular nucleophilic substitution
SCPx	Sterol carrier protein X-related thiolase
Ser	Serine
siRNA	Small interference ribonucleic acid
SNP	Single nucleoside polymorphism
t	Triplet
THC	Trihydroxycoprostanoyl
THF	Tetrahydrofuran
THP	Tetrahydropyran
tlc	Thin layer chromatography
TMS	Tetramethylsilane
Tol	Toluene
TPP	Thiamine pyrophosphate
Tyr	Tyrosine

UV	Ultra violet
VLCFA	Very long chain fatty acid
Vmax	Maximum velocity
Δ	Heat
µmol	Micromoles

For Mum and Dad

Mary Kirk

&

Steven Kirk

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Introduction to Prostate Cancer

Cancer is defined as a disease in which there is an uncontrolled proliferation of cells, typically with invasion and destruction of adjacent normal tissue. This often results in the metastasis of the cancer to distant parts of the body *via* lymphatic or circulatory systems. In 2007 cancer caused about 13% (7.4 million) of all human deaths.[2]

The prostate is a male reproductive organ which helps make and store seminal fluid. In adult men a typical prostate is about three centimetres long and weighs about twenty grams.[3]

Prostate cancer (PCa) is the most common cancer in men in the UK. It is responsible for a quarter of all new cases of cancer diagnosed in men. Worldwide, more than 670,000 men are diagnosed with prostate cancer each year. The highest rates are in the USA, Australia, New Zealand, Western and Northern Europe whilst the lowest rates are in East and South Central Asia (Fig. 1.01).[4]

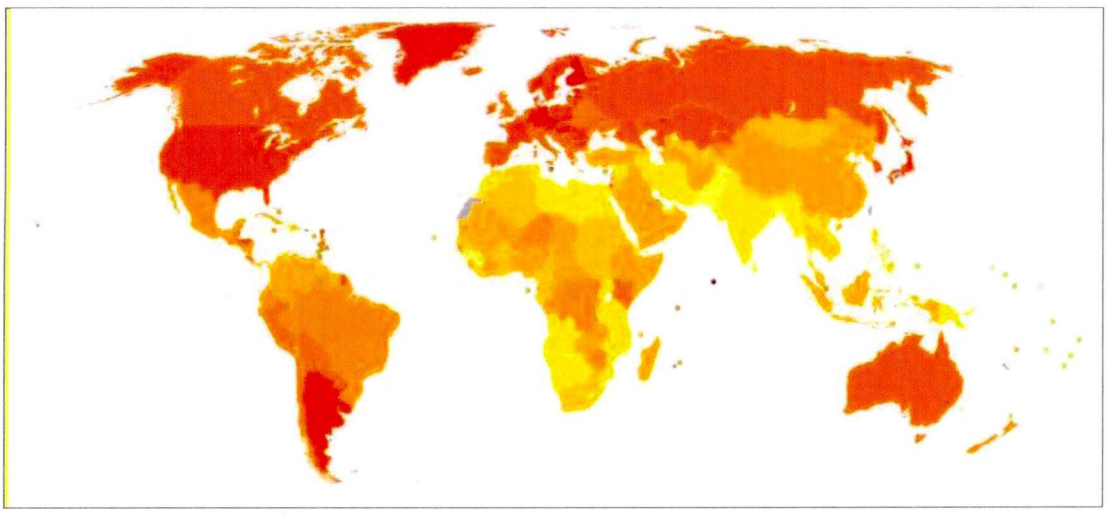


Fig 1.01. Age-standardised death rates from prostate cancer by country (per 100,000 inhabitants).[4]

Almost 60% of prostate cancer cases are diagnosed in men aged over 70 years and approximately one-third of men over the age of 50 have some cancer cells within their prostate.

1.1.2 History of prostate cancer

Prostate cancer was first described in 1536 by an Italian anatomist Niccolò Massa but it was not identified until some 300 years later in 1853.[5] From 1904 until the mid 20th century radical prostatectomy (removal of the prostate gland) was the main treatment.[6] In 1966 Charles B. Higgins, an American physician and physiologist won the Nobel Prize in Physiology and Medicine for the discovery of 'chemical castration'. Oestrogen was used to oppose testosterone production in men with metastatic prostate cancer.[7] In fact 'chemical castration' techniques are still used today, as will be discussed in Section 1.3.

1.1.3 Risk Factors

The strongest risk factor for prostate cancer is age, with very low risk in men under the age of 50 (Fig 1.02). Men with one or more first-degree relatives (father, brother, or son) diagnosed with prostate cancer have an increased risk of the disease. West African and Caribbean men have a higher risk of prostate cancer than Caucasian men, whilst men born in Asia have the lowest risk of the disease.

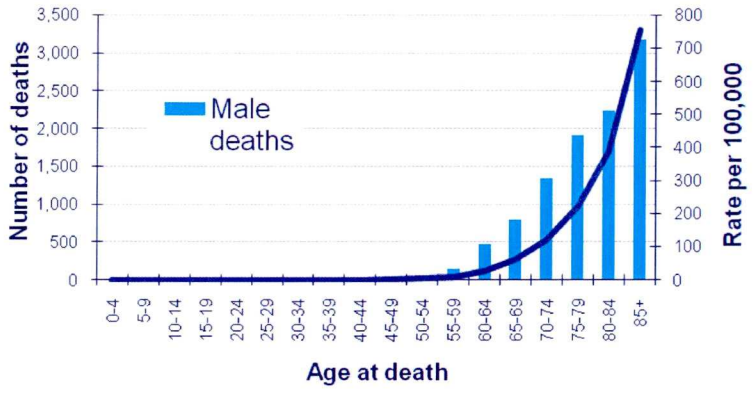


Fig 1.02. Graphical representation of prostate cancer mortality rate in relation to age.

1.1.4 Symptoms

PCa often has similar symptoms to that of benign prostatic hypertrophy (BPH), an enlargement of the prostate gland. These comprise of a range of urinal problems, including frequent urination (particularly at night), difficulty starting and maintaining a steady stream of urine, painful urination and haematuria (blood in the urine). Prostate cancer may also cause problems with sexual function, such as difficulty achieving erection or painful ejaculation.[8] Advanced prostate cancer may cause additional symptoms as the disease spreads to other parts of the body. The most common symptom is bone pain, often in the spine, pelvis or ribs[9] (due to metastasis to bone).

1.2 Progress in diagnostic markers.

The most routinely used PCa diagnostic marker is prostate-specific antigen (PSA). PSA is a serine protease, the physiological role of which is to liquefy seminal fluid.[10] Prostate cancer causes PSA to be released into the blood stream, increasing the plasma levels of PSA up to 10^5 fold.[11] Although there is no experimental data on the mechanisms of increased release, it is believed to result from the disruption of prostate integrity seen in prostate tumours, such as the loss of basal cell layer, ductal lumen architecture and epithelial cell polarity. High PSA levels (more than 4.0ng/ml) may indicate the presence of prostate cancer cells. However, in approximately 10% of prostate cancer cases, there is no rise in PSA above this threshold level, which creates a diagnostic problem for this group of individuals. PSA levels can be additionally increased by prostate infection, irritation, benign prostatic hyperplasia and recent ejaculation.[12, 13] Furthermore PSA levels can also appear reduced if the individual is on statin medication or suffers from obesity[14] – providing false negative results. A digital rectal examination (DRE) has been shown in several studies to also produce an increase in PSA[15]. If the levels of PSA are over 4.0ng/ml, a biopsy is usually taken for histological analysis to confirm the presence of malignant disease. Although PSA screening is practised in some parts of the world for early detection of prostate cancer, it is rejected in others because of the potential ambiguity of the results.[16]

There has been much interest in utilising other diagnostic markers, many of which rely on the absence of basal cells. These are cells which act as connective tissue between the luminal epithelium and the stroma. Basal cells form a continuous layer that encircles the entire

luminal epithelium which is absent in cancerous glands.[17] Some of the markers of interest include 34βE12, keratin 5/6, Bcl-2 and p63.

p63 is a homologue of the p53 tumour suppressor gene[18] and is thought to be involved in the development and maintenance of the prostatic secretory epithelial cell layer.[19] Keratin 5/6, normally expressed by complex epithelium is generally considered a marker of malignant mesothelioma (usually caused by exposure to asbestos). However, it has shown to be a demonstrable negative biomarker of prostatic, bile tract, and breast carcinomas.[20,21] Overall sensitivity, specificity, and diagnostic utility of keratin 5/6 in prostate biopsies is similar to that of 34βE12, which is a high molecular weight keratin.[20] Finally, Bcl-2 governs mitochondrial membrane permeability by regulating Ca^{2+}, pH, and voltage.

Diagnosing prostate cancer with basal cell biomarkers alone has its disadvantages. Not all benign glands are stained uniformly with basal cell markers and some show false negative staining if the individual has partial atrophy, atypical adenomatous hyperplasia or high grade prostatic intraepithelial neoplasia.[21,22] However, the latter two are often classed as 'precancerous'. Fig 1.03 demonstrates the difference in sensitivity and specificity between these negative biomarkers. 34 β E12 has the best sensitivity and specificity values (95% and 98%, respectively). The reliability of negative biomarkers in descending order is as follows: 34 β E12, p63, bcl-2, and keratin 5/6. From this data it is suggested that keratin 5/6 and bcl-2 should not be used to identify cancerous prostate cells, however 34βE12 could be used in conjuction with AMACR for increased reliability.

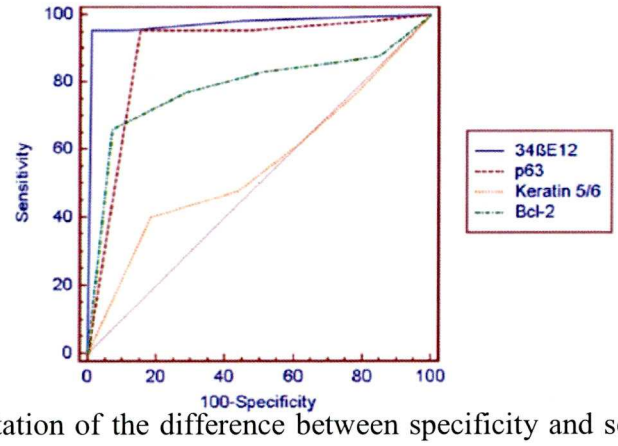


Fig 1.03. A graphical representation of the difference between specificity and sensitivity of different basal cell biomarkers for prostate cancer. 100-specificity represents the percentage of false positive results. [21]

More recently fatty acid synthase and Annexins have been investigated as diagnostic markers. Fatty acid synthase plays a key role in fatty acid biosynthesis and has been found comparable to α–methylacyl-CoA racemase (AMACR) in diagnosing malignant cells. However, there have been no studies performed to ascertain whether it is a negative stain for benign mimics of malignancy.[22] Annexins are a family of calcium binding proteins with the ability to bind phospholipids in the plasma membrane.[23, 24] Their function is to help organize exocytosis of intracellular proteins to the extracellular domain. In particular, Annexin II has shown to participate in ion channel regulation[25] and can inhibit cellular mobility. Annexin II is usually up-regulated in response to physiologic stress but has found to be underexpressed in prostate cancer.[26] However, it is suggested that Annexin II should be considered only as an adjunctive diagnostic marker because it can also stain some stromal cells and blood vessels. This can complicate interpretation of biopsy specimens by mimicking basal cells.[27]

Over the last decade AMACR has been thoroughly investigated and proved to be a viable and cost effective diagnostic marker for PCa. It is consistently over expressed in cancerous prostate cells and analysis of mRNA levels reveals an average up-regulation of ~9 fold in localized prostate cancer compared with normal and benign prostate.[28] This has been confirmed by real-time PCR analysis in addition to Western blotting and immunohistochemical analysis.[29] Several studies have also shown that AMACR is over-expressed in high-grade prostatic intraepithelial neoplasia[30-32], a precursor lesion for prostate cancer. AMACR is thus thought to be essential for early stage prostate cancer growth. The relative over expression of AMACR is strongest with localized prostate cells, followed by metastatic prostate tissue and benign prostate tissue having a negative expression of AMACR.[33]

AMACR detection is arguably a superior diagnostic marker than PSA.[34, 35] Its sensitivity has been reported as ranging from 82% to 100% and specificity has been reported as ranging from 79% to 100%.[36-38] PSA's respective sensitivity and specificity are 33% and 86%.[39] AMACR over-expression has also been shown in a number of other cancers, including breast, ovarian, lung, colorectal adenocarcinoma, papillary renal cell (kidney) carcinoma, melanoma and lymphoma (Fig 1.04). However, recent studies by Willemoe *et al.,* have shown that there is no discernible difference in AMACR expression between the malignant and non malignant cells in hepatocarcinoma.[40]

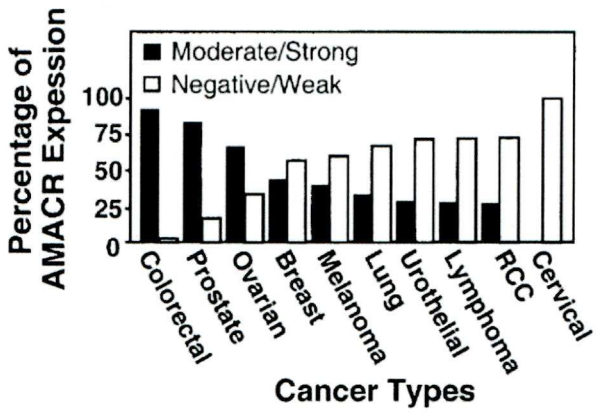


Fig 1.04. AMACR over expression in multiple tumours. AMACR protein expression was evaluated by immunohistochemistry on a multi-tumor microarray. The percentage of cases with positive staining (moderate and strong staining intensity) is summarized on the Y-axis.[41]

Positive staining for PCa with AMACR is not 100% accurate. There are reports of benign prostatic glands staining positive for AMACR[42-44] (0-21%). This has prompted experimentation with immunohistochemical cocktails of basal cell markers and AMACR. A triple immunohistochemical cocktail of 34βE12, p63 and AMACR gave a specificity of 100% with a sensitivity of 93.8%.[45] Earlier in 2010 Cetin *et al.,* reviewed five different diagnostic markers (34βE12, keratin 5/6, p63, bcl-2 and AMACR). Initial testing of the basal cell markers for staining benign glands demonstrated that p63 and 34βE12 were far superior to keratin 5/6 and bcl-2. Testing the same biomarkers with malignant glands gave the relative negative staining order as follows: p63>34βE12> bcl-2>keratin 5/6. They too noted that AMACR's expression could sometimes be deceiving, leading to misdiagnosis of adenocarcinoma.[43] It appears that there is no one omnipotent diagnostic marker that is universally accepted in the detection of prostate cancer. However, a combination of basal cell markers (such as 34βE12 or p63) with AMACR is a potentially compelling combination as a useful diagnostic strategy.

1.3 Current treatments

There are various treatment options for PCa. The first strategy for slow growing tumours is known as watchful waiting. The condition is monitored closely with routine check-ups and no physical treatment is performed. In some cases, particularly with older men, the patient will die of other causes rather than from prostate cancer.

The most common treatment for otherwise young, healthy men whose cancer is localised is to undergo surgery. The most common technique is a radical prostatectomy, whereby the whole prostate and some surrounding healthy tissue is removed. Recent developments in keyhole surgery (a laparoscopic prostatectomy), where the prostate is removed through smaller incisions and robot-assisted surgery has shown to be effective in minimising bleeding. This means less fatigue, and cardiovascular complications.

Conformal radiotherapy (CRT) allows for site specific radiotherapy that treats tumours which in the past might have been considered too close to vital organs and structures for radiation therapy. The tumour is scanned with X-rays to give a 3D image which is then sent directly into the radiotherapy planning computer. The computer program then designs radiation beams that 'conform' more closely to the shape of the tumour and avoid healthy tissue as far as possible. Side effects of this type of treatment may result in erectile dysfunction and difficulty in urinating.

High Intensity Focused Ultrasound (HIFU) is still an experimental form of treatment for prostate cancer. Beams are focused on the cancer tissue, causing the temperature within the tissue to rise to 65°- 85°C, destroying the diseased tissue by necrosis. Each beam treats a precisely defined portion of the targeted tissue which allows for non invasive ablation of the prostate. The most common problems reported after HIFU are infections in the prostate area and incontinence.

Cryotherapy is currently available for relapsing patients who have already undergone treatment with radiotherapy. Liquid nitrogen or argon gas is circulated through a cryoprobe, which is placed in contact with the tumour. Ultrasound or MRI is used to guide the cryoprobe and monitor the freezing of the cells, thus limiting damage to nearby healthy tissue. This type of therapy, as with HIFU and surgical methods, is limited to early localised cancer that hasn't

spread from the prostate. The main disadvantage of this type of therapy however is its long term effectiveness as it can miss microscopic cancer, which may lead to an additional relapse.

Hormonal therapy is the most widely used chemical treatment for prostate cancer. Testosterone (a hormone which increases the growth rate of the cancer cells) is prevented from reaching the cancer cells which limits the progression of the cancer and can even cause shrinkage. Testosterone and dihydrotestosterone (DHT) are the two major androgens in men, with testosterone present mainly in circulation, and DHT as the primary androgen in prostatic tissues. The pharmacological activity of both testosterone and DHT are reliant on the binding to androgen receptors (AR). DHT is regarded as the more potent of the two as it binds to ARs 5 times more strongly. The types of preventative testosterone production may include; removal of the testicles (orchidectomy), which directly removes the testosterone producing glands; injections of luteinizing hormone releasing hormone (LHRH) agonists such as Zoladex which control the production of testosterone; or administration of prostate specific 5α- reductase inhibitors such as finasteride (Proscar) which help prevent testosterone being converted to dihydrotestosterone. Dispite a rapid initial response in the majority of cases, PCa will ultimately progress to a hormone refractory stage, for which no curative therapies currently exist.[46] This is due to the remaining cancer still being driven by AR signalling (which stimulates mitogenesis). Abiraterone (which is currently in Phase III trials[47]) has received much attention as a selective irreversible CYP17A1 inhibitor. It has proven to significantly suppress cellular levels of dehydroepiandrostenedione (DHEA), androstenedione, and testosterone.[48] Phase II trials indicate that abiraterone shrinks the tumour and may reduce PSA levels[49] with low levels of toxicity.

The selection of treatment options may be a complex decision involving many factors, including age, health, the severity of cancer and possible side effects.

1.4 α-Methylacyl-CoA Racemase and its Function.

1.4.1 AMACR' physiological role

AMACR is intrinsically involved in the β-oxidation pathway of branched chain fatty acids. It is a peroxisomal and mitochondrial enzyme which is found in both prokaryotic and eukaryotic organisms. It has a wide substrate specificity and its natural role is to catalyse the interconversion of (*R*) and (*S*) stereoisomers at the α-position of branched chain acyl-CoA esters such as pristanoyl-CoA (**8**) (which is acquired from meats and dairy products) and trihydroxycholestanoyl-CoA (a bile acid intermediate). This provides the required (*S*)-configured substrate for the stereospecific *acyl-CoA oxidase.*[50]

Fatty acid β-oxidation is a universal property of peroxisomes in most, if not all, organisms. In yeast and plants, peroxisomes are the sole site of fatty acid β-oxidation, whereas in higher eukaryotes β-oxidation may occur in both mitochondria and peroxisomes. Its function is to break down fatty acids by the sequential removal of two carbon units, leading to the formation of acetyl-CoA or various derivatives which enter directly into the Krebs cycle. The β-oxidation pathway involves dehydrogenation, hydration, dehydrogenation again, and thiolytic cleavage (Fig 1.05). For every one cycle of β-oxidation, 14 ATP molecules are produced[51].

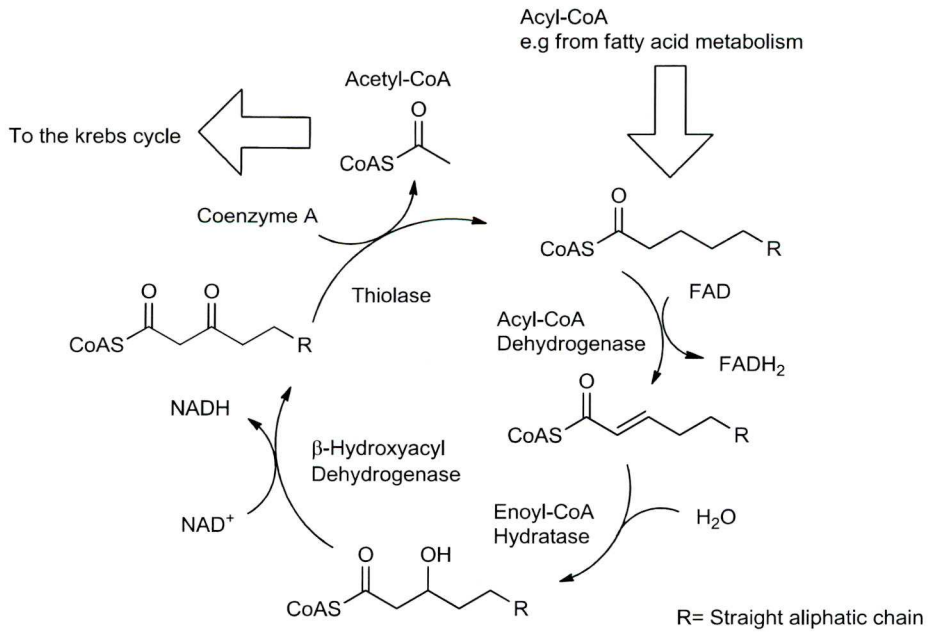


Fig 1.05. The β-oxidation cycle of straight chain aliphatic acids.

The major differences between peroxisomal and mitochondrial β-oxidation are the different substrate specificities and transport of substrates/products of β-oxidation across the membrane.[52] Short and medium-chain fatty acids are exclusively β-oxidised in mitochondria. The β-oxidation pathway of VLCFA (very long chain fatty acids), 2-methyl branched-chain fatty acids (such as pristanic acid) and the bile acid intermediates dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA) are primarily located in the peroxisome (Fig 1.06).

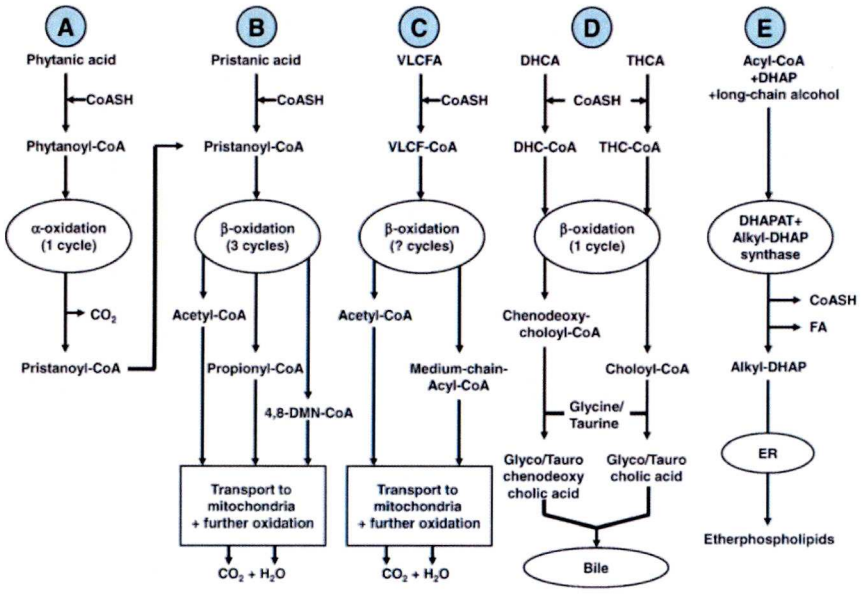


Fig 1.06 Schematic representation of the pathways of lipid metabolism in peroxisomes.[53]

Pristanic acid (**7**) is either directly ingested through consumption of meats and dairy products, or it is metabolised from phytol (**2**)/phytanic acid (**4**). Phytanic acid (**4**) can be acquired by conversion of phytol (**2**) (from digestion of plant material (**1**)) to (*E*)-phytenic acid by the successive action of a currently unknown *alcohol dehydrogenase* and *fatty aldehyde dehydrogenase* (FALDH). Phytenic acid is then activated by a synthetase to produce (*E*)-phytenoyl-CoA and finally converted by an *enoyl-CoA reductase* into phytanoyl-CoA (Fig 1.07).

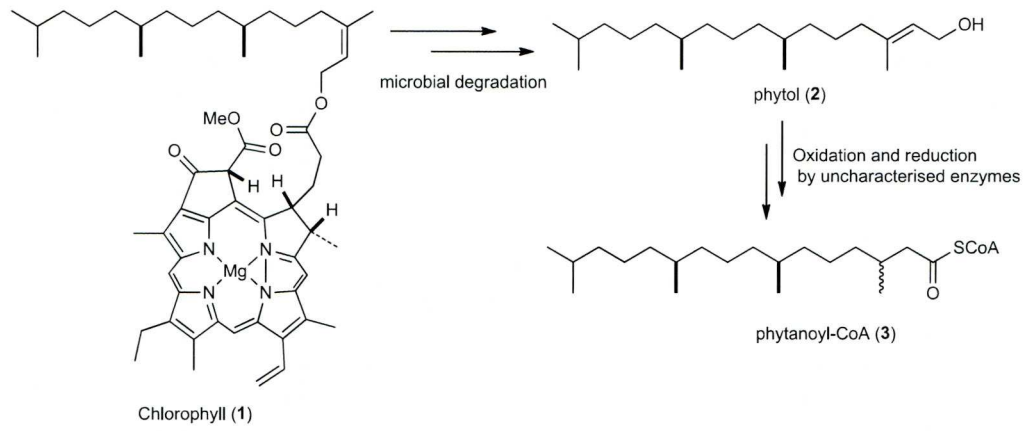


Fig 1.07. Acquisition of phytanoyl-CoA from chlorophyll adapted from Mukherji *et al.*[54]

Digested phytanic acid is normally α-oxidised through shortening of the alkyl chain by one carbon atom, yielding pristanic acid (**7**) and CO_2 (Fig 1.08). Studies have shown that peroxisomal α-oxidation is not a stereoselective process, so that after the oxidation of phytanic acid (**4**), both (2*R*,6*R*,10*R*,14)- and (2*S*,6*R*,10*R*,14)-pristanic acid (**7**) are formed.[50]

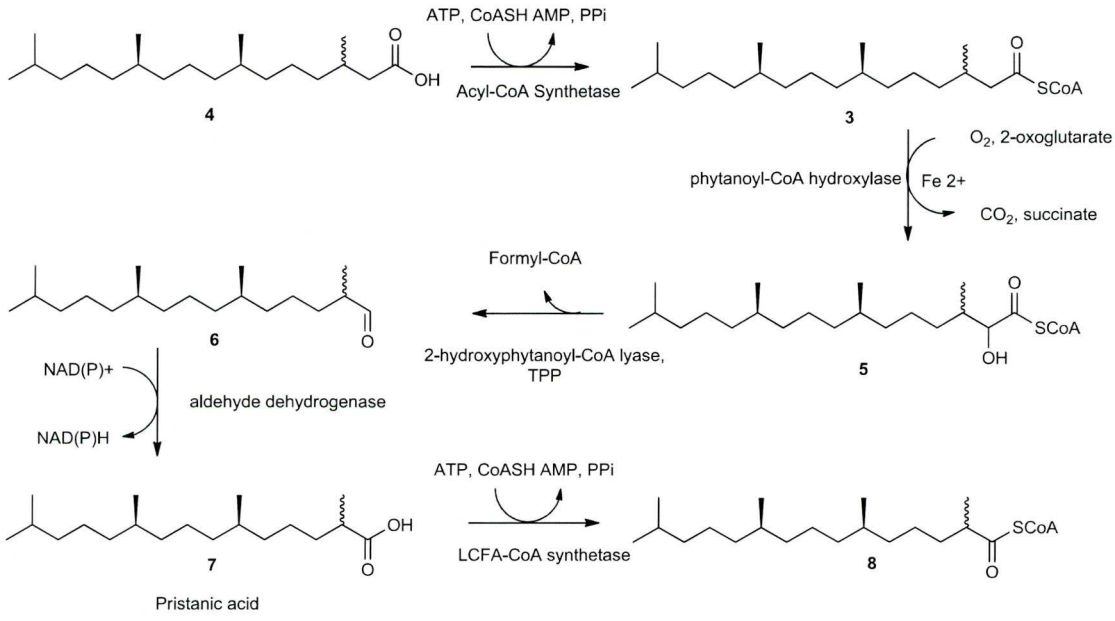


Fig 1.08. The α-oxidation pathway of phytanic acid.

Upon CoA thioesterification, pristanoyl-CoA (**8**) undergoes 3 cycles of β-oxidation in peroxisomes[55] to produce two propionyl-CoA (**19**) molecules, acetyl-CoA (**20**) and 4,8-dimethylnonanoyl-CoA (**21**). This is then transported to the mitochondria where it is fully oxidised.[55-57] There are two possible routes in which the peroxisomal metabolite is transported into the mitochondria. The first is the hydrolysis of the CoA thioester to form 4,8-

dimethylnonanoic acid, transfer across the peroxisomal membrane (by a currently unknown transporter) and finally transfer into the mitochondria in its protonated form where it is then further oxidised.[58]

The second possible route for mitochondrial entry is conversion of the CoA ester to a carnitine ester within the peroxisome, where it is then carried across the peroxisomal membrane and then transported into the mitochondria by mitochondrial carnitine/acylcarnitine carrier (CACT).[56, 59] Once inside the mitochondria, the carnitine ester will be re-CoA esterified and undergo further oxidation (Fig 1.09).

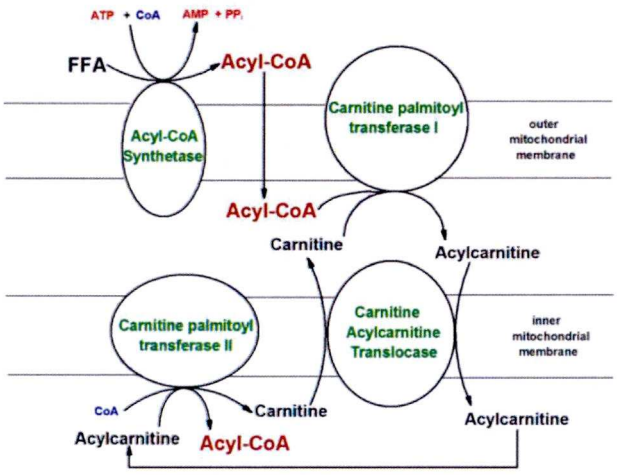


Fig 1.09. A representation of a possible route of entry for short branched chain fatty acids into the mitochondria.[60]

In the liver, the bile acid intermediates DHCA and THCA only undergo one cycle of β-oxidation in peroxisomes, with choloyl-CoA and chenodeoxycholoyl-CoA as end products respectively. These two CoA-esters are then conjugated with either taurine or glycine within the peroxisomes. Subsequently, the taurine and glycine esters are transported out of the peroxisome into the cytosolic space, followed by transport across the canalicular membrane, ending up in bile.[61, 62]

The penultimate enzymatic manipulation before β-oxidation is the racemisation of the α-methyl group by AMACR. The first step of the β-oxidation cycle is catalyzed by acyl-CoA oxidases (ACOX) which are flavoproteins. These enzymes generate H_2O_2 as a product to produce a *trans* enoyl-CoA ester. In humans there are two main acyl-CoA oxidases (ACOX1 and ACOX2). ACOX1 is specific for VLCFA-CoA, whereas ACOX2 preferentially reacts

with the CoA-esters of 2-methyl branched-chain fatty acids, including pristanoyl-CoA (**8**), DHC-CoA and THC-CoA (**9**)[63] and only accepts α- methylated substrates in the (*S*) form. The next two steps in β-oxidation are catalyzed by one of two bifunctional proteins harbouring two separate enzymatic functions: enoyl-CoA hydratase and 3-hydroxy-acyl-CoA dehydrogenase activity.

The first of these two bifunctional proteins, multifunctional enzyme 1 (MFE-1 - also referred to as L-specific bifunctional protein, LBP) generates 3-ketoacyl-CoAs (**12**) *via* a (3*S*)-hydroxyacyl-CoA (**13**) intermediate. The other bifunctional protein, multifunctional enzyme 2 (MFE-2, also referred to as DBP) has a (3*R*)-hydroxyacyl-CoA-ester as an intermediate (**11**).[64] (*E*)24-ene-THC-CoA (**10**) is normally hydrated to (24*R*,25*R*)-24-OH-THC-CoA (**11**) by MFE-2, but can also be converted to (24*S*,25*S*)-24-OH-THC-CoA (**13**) by the hydratase unit of MFE-1.[65] (24*S*,25*S*)-24-OH-THC-CoA (**13**) is, however, not a substrate for the dehydrogenase unit of MFE-1 or MFE-2.[65, 66] but AMACR can convert it to the (24*S*,25*R*)-isomer (**14**)[67] which can be oxidised by the dehydrogenase unit of MFE-1 (Fig 1.10).

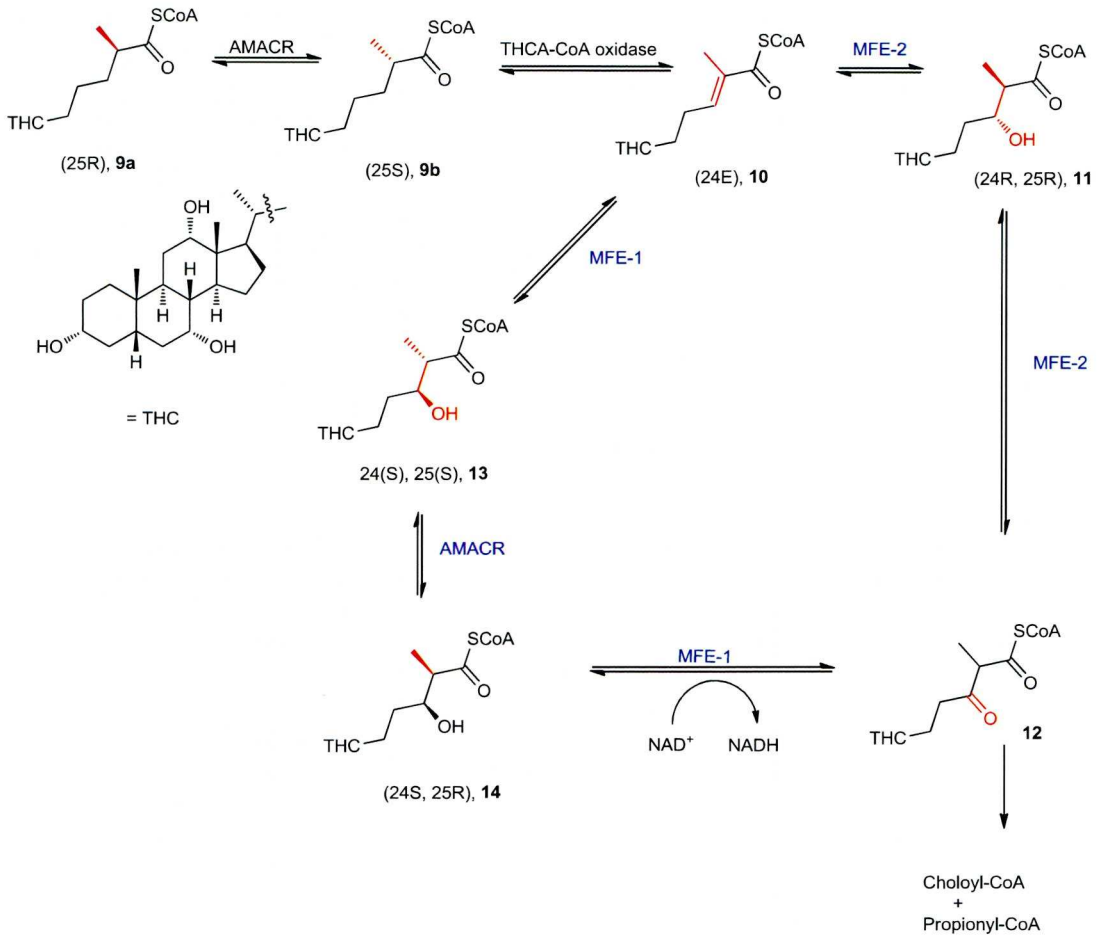


Fig 1.10. A Schematic representation of the function of multifunctional enzyme 1 and 2 in the β-oxidation pathway.

The final chemical manipulation of the β-oxidation pathway is the thiolytic cleavage to release an acetyl-CoA thioester or derivative (Fig 1.11). There are two main thiolases: A straight chain 3-oxoacyl-CoA thiolase (ACAA1) and a sterol carrier protein X (SCPx). SCPx is reactive with the 3-ketoacyl-CoA esters of pristanic acid, DHCA and THCA whereas ACAA1 only accepts the 3-ketoacyl-CoA esters of VLCFA.[68]

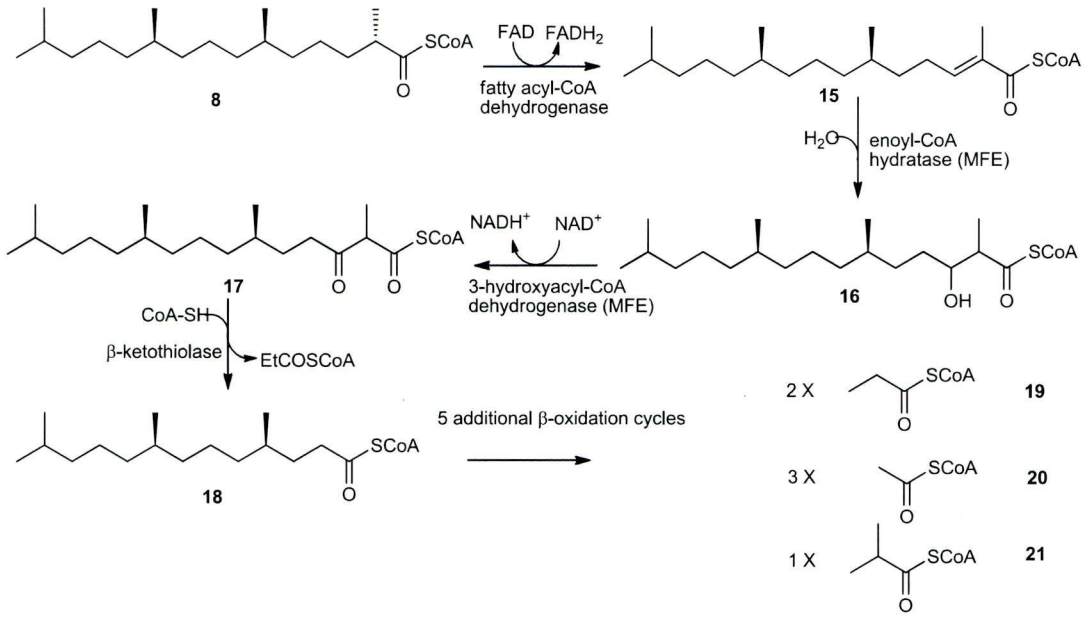


Fig 1.11 A Schematic representation of the β–oxidation pathway.

1.4.2 Significance of AMACR deficiency

All defects of peroxisomal β-oxidation involve the nervous system which can include Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease phenotypes. In contrast, AMACR deficiency appears to be a milder disorder. To date, only 6 patients (4 adults and 2 children) with documented α-methylacyl-CoA racemase deficiency have been reported.[69] Symptoms seem to be mostly delayed, developing in adulthood such as adult-onset sensory motor neuropathy. This is a rare, congenital disorder whereby an individual's inability to utilize AMACR results in an accumulation of branched-chain fatty acids.[3, 70] The phenotypical expression of this condition is relapsing encephalopathy, seizures and cognitive decline.[71] In one patient, AMACR deficiency lead to retinitis pigmentosa, primary hypogonadism, epileptic seizures, and a widespread axonal neuropathy.[72] It has also shown to produce demyelinating polyneuropathy. Symptoms in children can include fat soluble vitamin deficiency (K,E,D) and cholestatic liver disease.

Studies carried out on patients with congenital deficiencies of palmitoyl-CoA oxidase[73], D-bifunctional protein[73] and β-keto-thiolase[73] respectively show symptoms which include severe dismorphic features, neuromuscular seizures, and cardiac defects. In all cases, death occurred before four years of age. This effectively singles out AMACR as the only viable branched chain metabolising enzyme that could be targeted, without the risk of severe side

effects. Additionally in many cases, dietary modification and/or bile acid supplementation offer an effective treatment for the symptoms of AMACR deficiency.[74]

1.4.3 Splice variants and polymorphisms of AMACR

Mubiru *et al.*, have cloned six different forms of AMACR transcripts from cDNA derived from the PC-3 human prostate cell line. These include AMACR IA, IB, IIA, IIAs IIB and AMACR IA_{DEL}. AMACR 1A is the major isoform of AMACR, which contains the putative KASL peroxisomal targeting sequence. AMACR IB contains the same sequence with AMACR IA, but with exon 3 of the gene omitted. AMACR IIA shares the first four exons with AMACR IA but uses an alternative fifth exon. AMACR IIAs is an alternative version of AMACR IIA by a shortening of exon 5. AMACR IIB also has an alternative exon 5 and lacks exon 3. This splice varient has a surprisingly high homology with fumarate hydratase.[71] $AMACR_{DEL}$ has an alternative splicing event in the 5th exon of the gene which causes a shift in the reading frame. All splice variants have the KASL peroxisomal targeting sequence missing and are believed to target the mitochondrial organelle.[75]

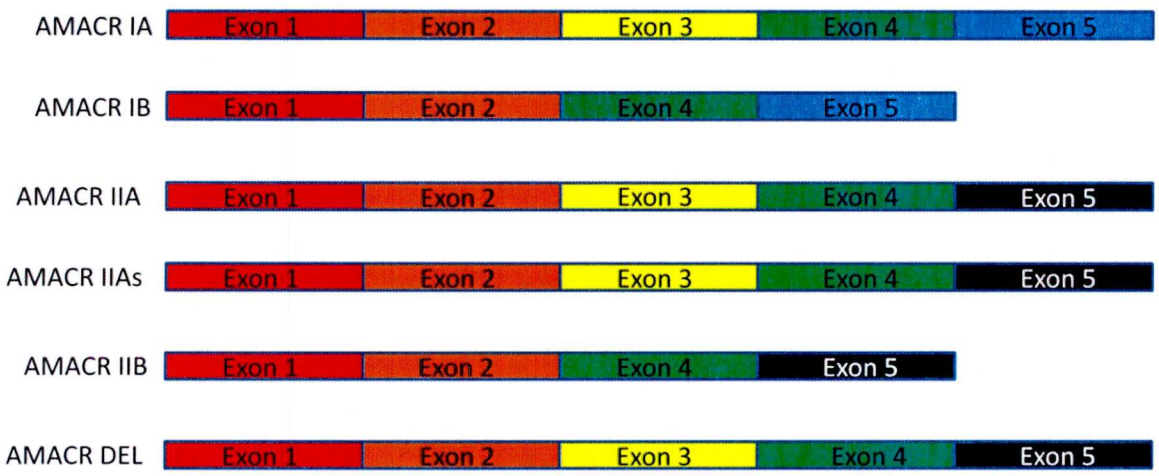


Fig 1.12 Schematic representation of the known AMACR splice variants. Exons are colour coded to represent identical sequences. Exons that are represented by the colour black represent a deviation from the major isoform AMACT IA. All variations of exon 5 differ from each other as described above. Exons that are omitted in the scheme, are also absent in the genetic sequence. Exons. Taken from *Gene* **2004**, *327*, 89-98 and *Prostate* **2005**, *65*, 117-123.

At present, the potential biological significance of different AMACR splice variants remains unclear as all splice variants are upregulated in PCa.[71, 76] However, studies on the two major variants of AMACR (AMACR 1A and IIA, resulting from the alternative use of the last exon) has showed that AMACR 1A is quantitatively up-regulated in prostate cancer compared with normal prostatic tissues, whereas AMACR IIA seemed to be expressed only in prostate cancer.[77] This indicates that AMACR IIA is only expressed when the cell becomes cancerous.

Studying the difference in allele frequencies between hereditary prostate cancer (HPC) and controls has shown a variation at four polymorphic regions in the AMACR gene (M9V, D175G, S201L, K277E).[78] The M9V polymorphism seems particularly associated with increased risk of prostate cancer.[79] A rather compelling study that investigated a connection between regular Ibuprofen users and AMACR expression found that risk for prostate cancer was significantly reduced among regular Ibuprofen users who carried the aforementioned allele variants (M9V, D175G, S201L, and K277E[80]). It may be inferred that AMACR plays a role in converting Ibuprofen from its COX-inactive ((*R*)-enantiomer) to the COX-active form ((*S*)-enantiomer) (as will be explained in results and discussion). Both AMACR and COX-2 are overexpressed in prostate cancer.[81] Ibuprofen is a substrate/competitive inhibitor of AMACR and a competitive inhibitor of COX-2. COX-2 is involved in the production of prostaglandins which are involved in the regulation in cell growth and inflammatory responses. It is possible that ibuprofen has a dual anticancer effect by inhibiting both these enzymes. Alternatively the reduced incidence of cancer in regular Ibuprofen users with these SNPs may also be due to an increased turnover of the pharmacologically active form of ibuprofen, which could have increased efficacy on the COX-2 enzyme.

Although there is a link between these SNP's with hereditary PCa, there is no evidence to suggest that they play a significant role in the development of sporadic prostate cancer.[80]

1.5 Mechanism of AMACR

1.5.1 Substrates and substrate specificity

AMACR accepts a wide range of CoA esterified substrates (Fig 1.13) which include bile acid intermediates (di- and trihydroxycoprostanoic acid (**9**)), the non-steroidal anti-inflammatory metabolite – ibuprofenoyl-CoA (**22**), and branched chain fatty acids (with a minimum chain length of 8 carbons).[67, 82-84] The coenzyme A thioester activation of these substrates is essential for enzymatic activity, as the free acid analogues do not racemise[82]. [2,3-^{3}H] Palmitoyl-CoA and [2,3-^{3}H] phytanoyl-CoA are inactive.[82] This suggests there is an inherent requirement for an α- methyl group as neither 3-methyl-branched chain acyl-CoA's nor linear-chain acyl-CoA's are accepted. More recently however, Lloyd *et al.,* have demonstrated that straight-chain fatty acyl-CoA esters are also able to undergo deprotonation and reprotonation events.[85] Nonetheless, the straight chain acyl-CoA esters undergo proton exchange at a significantly lower rate than their α-methylated analogues.

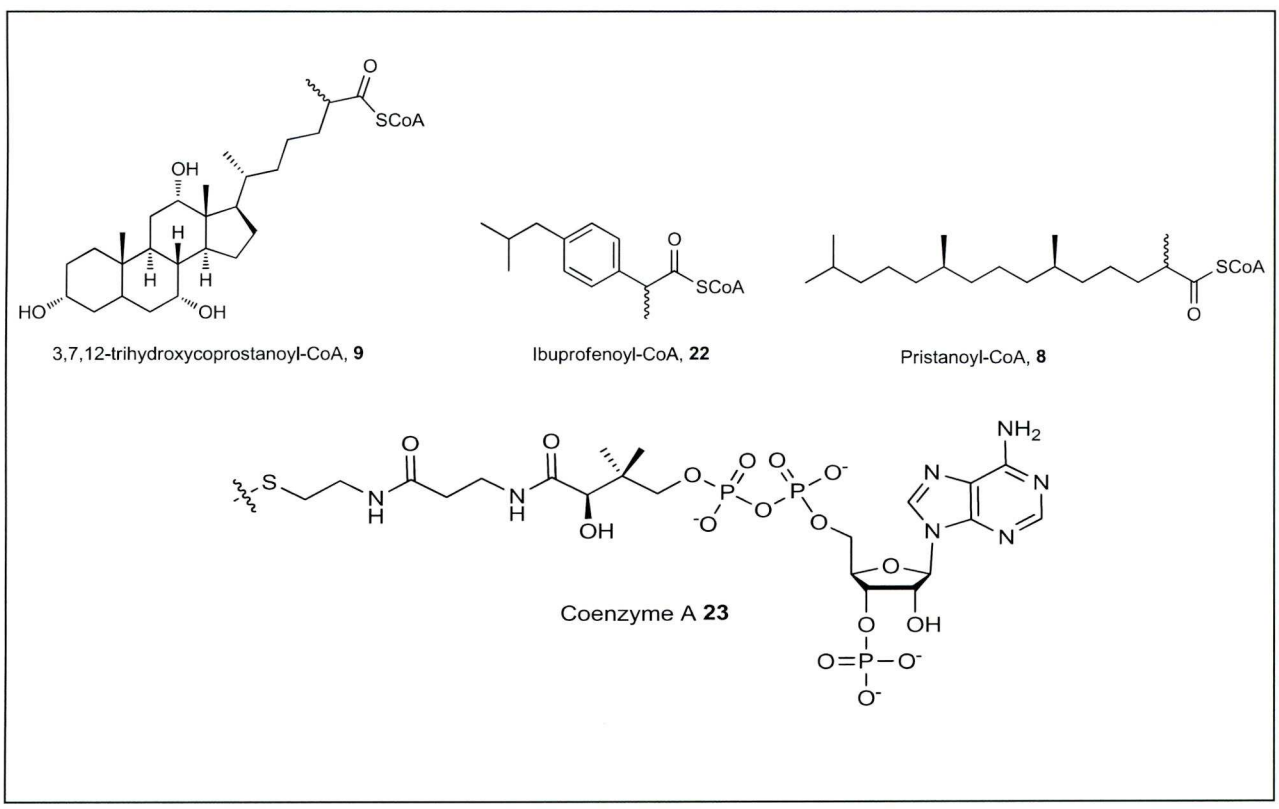


Fig 1.13 Substrates of AMACR with a representation of the SCoA moiety.

1.5.2 Evolution of AMACR

The current closest amino acid sequence to the human AMACR enzyme was found in protein Q5RF52_PONAB from *Pongo abelii* (Sumatran orangutan). This protein is very similar to human AMACR, with a remarkable 376 identical and 8 similar amino acids resulting in 97% identity (acquired from the German cDNA consortium, 2004 *via* ncbi). MCR (*Mycobacterium tuberculosis* racemase), also has a high sequence identity with mouse, rat and human AMACR of 41, 44 and 43% respectively, with 153 out of 353 common amino acids[86]). To date, MCR is the only fully characterised enzyme of this family. L-carnitine dehydratase (CaiB) also shares a large degree of similarity with AMACR. This enzyme was thought to convert L-carnitine to 4-(trimethylammonio)but-2-enoate through the abstraction of an α–proton but according to Heider's review, it is now thought to catalyse CoA transfer between carnitine and amino crotonate.[87]

AMACR, MCR and L-carnitine dehydratase belong to the family III CoA transferases also referred to as the CAIB-BAIF family.[88] CoA transferases are found in organisms throughout nature. Nominally CoA transferases catalyse reversible transfer of coenzyme A groups from CoA thioesters to free acids. There are at least three families of CoA transferases, which differ in sequence and reaction mechanism. The first structural studies of formyl-CoA transferase (FRC)[89, 90], a type III CoA transferase, revealed that they are a superfamily of interlocked dimers.

The Class I CoA transferases, such as succinyl-CoA transferase[91] and butyryl-CoA transferase[92] are a group of enzymes primarily involved in fatty acid metabolism and have a well established 'ping pong' mechanism. A CoA donor (**24**) (normally succinyl-CoA or acetyl-CoA) forms a covalent intermediate involving a glutamate residue to form a glutamyl-acyl anhydride intermediate (**25**). This undergoes nucleophilic attack from the released CoA moiety to liberate a free acid and form a γ-glutamyl-CoA thioester (**26**). A second free acid (3-oxo acids or 3-keto acids) then attacks this thioester (**26**) which ultimately forms a molecule of 3-oxo(keto)acyl-CoA (28) via another anhydride intermediate (**27**) and reactivates the enzyme[93, 94] (Fig 1.14).

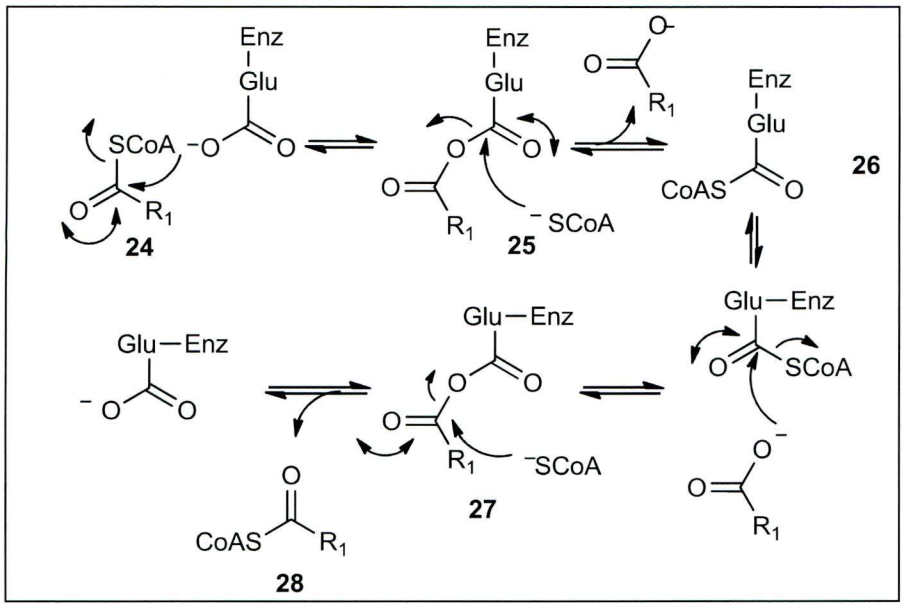


Fig 1.14 A Schematic representation of the mechanism of action for the type I CoA transferases.

The smaller group of Class II CoA transferases catalyzes a partial reaction in the citrate and citramalate lyase complexes.[87] These reactions do not include covalent enzyme intermediates, but transfers a covalently bound 3'-dephospho CoA molecule (bound to an acyl carrier protein (ACP) in the enzyme complex) between two acids (**29**, **31**) *via* a mixed anhydride intermediate (**30**).[95, 96] In both classes of CoA transferases, the reactions involve formation of thioester and anhydride intermediates, but the mechanistic details differ considerably (Fig 1.15).

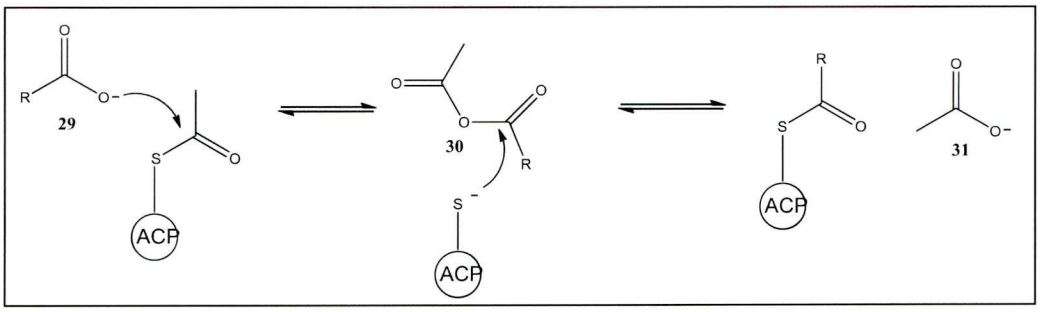


Fig 1.15. A Schematic representation of the mechanism of action for the type II transferases.

Class III CoA transferases are not consistent with a 'ping-pong' mechanism as in the Class I CoA transferases but form covalently enzyme bound intermediates (unlike the class II transferases). In the case of formyl-CoA transferase the reversible mechanism proceeds through a possible ternary complex, whereby formyl-CoA is covalently bound to an Asp side chain to form an anhydride intermediate (**33**). Again, the free thio attacks this anhydride to form an enzyme bound thioester (**34**). This is followed by nucleophilic attack of oxalate to form a covalently bound oxalate anhydride intermediate (**35**). Finally, cleavage from the enzyme by CoASH forms oxalyl-CoA (**36**) and formic acid (**37**) (Fig 1.16).[96, 97]

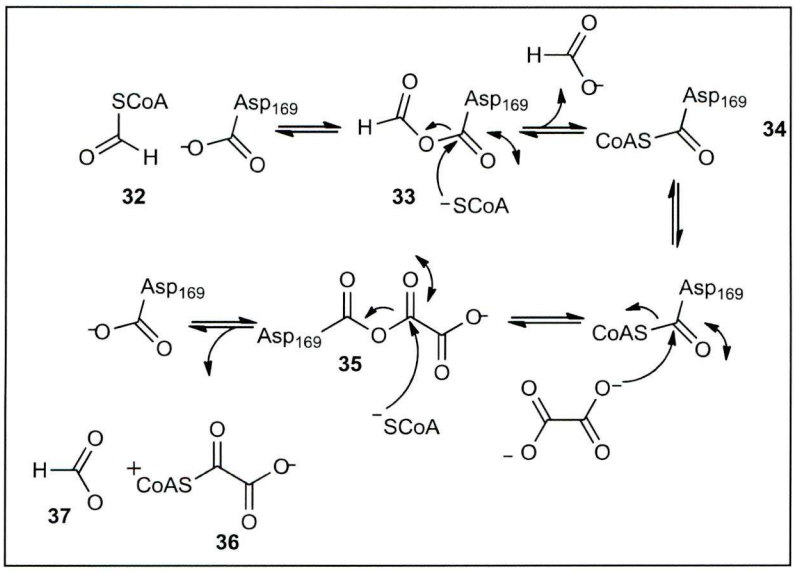
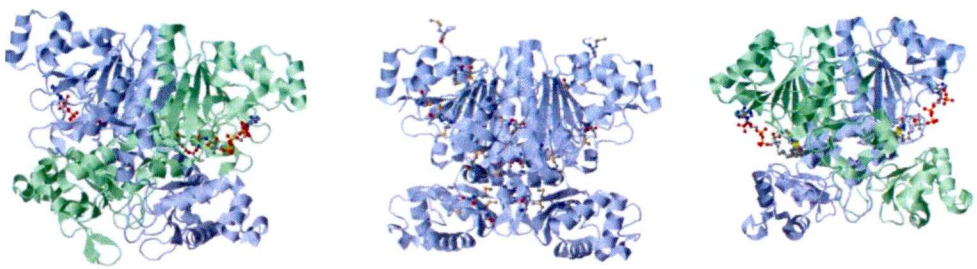


Fig 1.16. A Schematic representation of the mechanism of action for Formyl-CoA transferase, an extensively studied type III transferase.

MCR shares a 24% sequence identity with FRC. The quarternary structures of the interlocked dimers are very well preserved (Fig 1.17). The tertiary structure of the large domain is also well conserved. However, the helical part of the small domain differs[98] in the loop which connects its large domain to its small domain and provides residues which are important for the binding of the acyl moiety. The corresponding loops of formyl-CoA transferase, carnitinyl-CoA transferase and MCR are of variable lengths.[61] Due to the high sequence and quaternary structural similarities between FRC and MCR, some have proposed that AMACR is possibly a bifunctional enzyme that activates (by CoA thioesterfication) and epimerises its substrates[87] in a similar manner to FRC.

Although the predicted mode of CoA binding appears to be similar in both enzymes, that both use an Arg residue to provide stacking interactions with adenine (Arg38 with FRC, Arg85 with MCR), a Lys residue interacting with the pantethine moiety (Lys137 and Lys62[61] respectively) and similar key catalytic residues (Asp169 as a nucleophile for FRC and Asp156 as a base for MCR), it is unlikely that AMACR is bifunctional.

Free acids are not accepted as substrates of AMACR[82] and are known to be independently CoA thioesterfied by *long chain acyl synthetase.*[99] Additionally, the substrate specificities are very dissimilar. In MCR, the binding pocket is much more extended and exposed to solvent, due to shorter loop regions near the active site. This fits in with the findings that AMACR has a wide degree of substrate specificity with respect to its side chain (steroidal, aromatic, and pristanoyl based moieties). FRC however is very specific towards small polar molecules. Superfamily members often share key catalytic site components that have the same chemical function across the superfamily. This common aspect often leads to a degree of mechanistic similarity. However, this is not the case between class III CoA transferases and AMACR. The respective catalytic residues catalyse different reactions employing distinct chemistry.



Formyl-CoA Transferas *Carnitinyl-CoA transferase* *2-Methylacyl-CoA Racemase*

Fig 1.17. X-ray crystal structures of three enzymes belonging to the type III transferase superfamily taken from pdb.com orientated to show the high degree of structural similarity.

1.5.3 Affinity and binding.

Structural and kinetic data of mutated variants of MCR has provided strong evidence that the residues Asp156 and His126 are crucial for the catalytic racemisation.[61] The chiral α-carbon is tightly anchored between these residues with a distance of 3.7 and 3.3Å respectively. These active site catalytic residues are conserved in human AMACR 1A. Glu241 and Asp152 are also key residues, which stabilise the catalytic bases through hydrogen bonding. Due to the proximity of the catalytic residues in relation to the functional carbon, a two base mechanism is hypothesised (Fig 1.18).

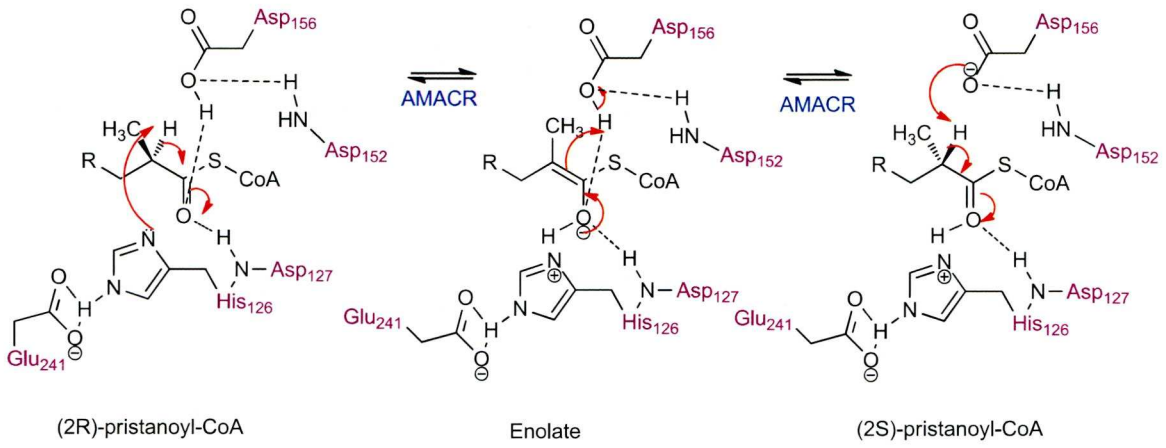


Fig 1.18. Possible mechanistic pathway for racemisation by MCR.

X-ray crystallographic data of MCR has shown the 2-methyl acyl-CoA molecules hydrogen bond with their thioester oxygen to the N-H on the Asp127 residue.[61] As previously stated, adenine on the CoA moiety of the substrates has a stacking interaction with the side chain of Arg38. The 3'-phosphate moiety is salt bridged to Lys62 and Arg91 and the pyrophosphate moiety is salt bridged with Arg85. The 2-methyl group contacts side chain atoms of His126, Asp127, Asp156, Leu217 and Tyr 224. During the 1,1-proton transfer, the acyl moiety (of Ibuprofen) moves over a methionine rich hydrophobic surface (Met50, Met198, Met199, Met202, Met207 and Trp208), contacting Ile16, Pro20, Met50 and Met198 as the (*S*)-enantiomer, and contacting Met216, Leu217 and Ile240 as its (*R*)-counterpart. In general, the inversion of the chiral center does not change the manner in which the substrate binds. The α-carbon has a tendency to point towards Asp156 in its (*R*) form, whereas the analogous enantiomer points towards His126. The 2-methyl group moves slightly but remains bound in the same pocket and is an important group for the affinity of the substrate to the enzyme. The

CoA moiety remains rigidly bound, and the acyl group glides over the large hydrophobic pocket.[61]

1.5.4 Mechanism

Incubation of [2-^{3}H]pristanoyl-CoA and [24, 25-^{3}H]trihydroxycopristanoyl-CoA with rat AMACR has shown it to catalyze a rapid exchange of the α-tritium against a proton from water. This is highly indicative that the mechanism involves abstraction of a proton at the α-position.[82] It was thus assumed that the enzyme functions by proton abstraction to form a stabilised enol(ate) in a mechanism similar to methylmalonyl-CoA racemase.[100] This is further supported by the fact that the pKa of the α-proton significantly decreases from ~34 to 21 with the thioesterification of a free acid.[101] This enables the otherwise relatively strong C-H bond to succumb to cleavage under mild enzymatic conditions.

More recently, mechanistic studies by Darley *et al.*, on the recombinant human AMACR using NMR have suggested a planar intermediate.[102] This study also showed that the deprotonation occurred with either enantiomer followed by a non-stereospecific reprotonation of the planar intermediate, proving that AMACR is a true racemase.

The aforementioned MCR X-ray crystallographic data has also shown that once a substrate is bound to the enzyme's active site, the thioester oxygen atom and the 2 methyl group of the substrate are in a *cis* conformation with respect to each other, and both point inwards towards the bottom of the catalytic pocket. These results are consistent with findings from Darley *et al.*, that the transition state consists of a planar intermediate.

A planar intermediate is not direct evidence of the existence of an enol(ate) and so two possibilities can be postulated. The first is a ketene intermediate (Fig 1.18), whereby the proton abstraction would temporarily dissociate the coenzyme A moiety from its parent molecule. The second is the aforementioned enol(ate) intermediate (Fig 1.18/1.19) which is stabilised by the enzyme and is the proposed intermediate by which related enzymes catalyse their reactions.[75] There is no literature precedent for a ketene intermediate in reactions catalysed by a racemase and so it is highly probable that the mechanism of action goes *via* the enol(ate). However, one must not forget that FRC performs its catalytic activity by the release and reattachment of the CoA moiety. A carbanion intermediate (Fig 1.19) is unlikely as it

would provide a tetrahedral intermediate which would not facilitate inversion of configuration and would also contradict the established evidence for a planar intermediate.

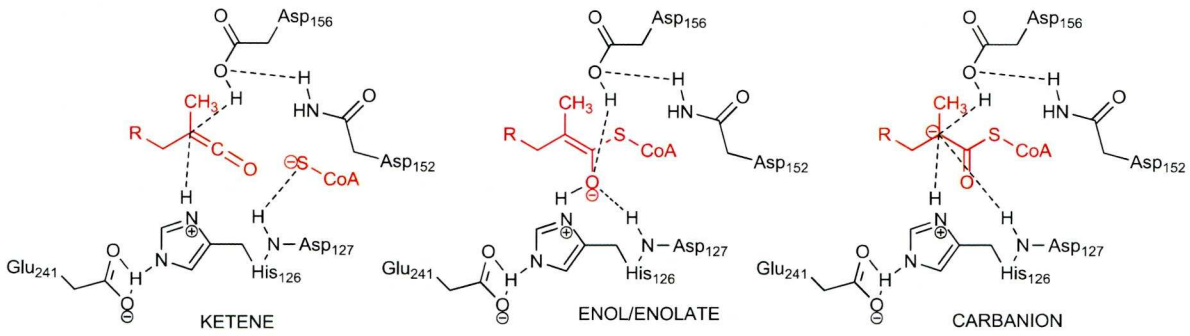


Fig 1.19. A Schematic representation of the possible enzymatic intermediates adapted from Darley *et al.*[102]

Incubation of AMACR in D_2O with non deuterated substrates[102] has shown a higher rate of deuterium incorporation than that of known two base racemases such as mandelate racemase,[103] methylmalonyl-CoA epimerase[104] and diaminopimelic acid epimerase.[105] Additionally, it was noted that AMACR had no preference in rate of deuterium incorporation for either enantiomer. This is more representative of a classical one base mechanism[106] and is exemplified with the incubation of (*S*) 2-methyldecanoyl-CoA with AMACR in D_2O. Quenching and analysing the reaction during the initial burst furnished incorporation of deuterium at the α-position into both enantiomers. This is a standard method of assessing whether the enzyme has a mono or dual base catalytic racemisation system.[107] If the enzyme racemises the substrate by utilizing two bases, then terminating the reaction during the initial burst would provide the (*R*) 2-methyldecanoyl-CoA in its α-deuterated form, but would have kept the (*S*) 2-methyldecanoyl-CoA protonated. This is due to the inability of the monoprotic bases to exchange a proton with the solvent during the lifetime of the enol(ate). AMACR is different. In most racemases utilising two bases, the catalytic active site is locked away from the bulk solvent and deuterium incorporation can be limited.[103] The active site of AMACR however resides nearer the surface of the enzyme (Fig 4.13) as is typical of the type III transferases[61, 90] and so will have a higher propensity to interact with the bulk solvent.

Further studies on mutated variants of the enzyme should be conducted to unequivocally ascertain whether the enzyme functions as a mono or dual base racemase. However, due to

the proximity of Asp156 and His126 and the relative orientation of the substrate in its different enantiomeric state, it is difficult to imagine that it functions by using only one of these bases.

To summarise, despite MCR/AMACR belonging to the type III transferase family, MCR/AMACR probably functions as a two base racemase by deprotonating/reprotonating the α-proton from a 2-methyl CoA thioester *via* an enzyme stabilised enol(ate) intermediate. This is the only known racemase of this family. Moreover its substrate specificity is surprisingly distinct from the formal type III transferases, which catalyse the transfer of the CoA moiety between specific small, polar acyl groups.

1.6 The human AMACR model based on MCR homology

Dr Robert Gibson from the University of Liverpool has built a model of human AMACR based on the crystal structure of MCR obtained from *Mycobacterium tuberculosis*. This was achieved by initially performing a blast search to check whether the key catalytic residues are conserved. As previously stated, MCR and AMACR share 42% sequence identity. From this we can be confident that the fold of the protein is very similar. Since the key catalytic residues and enzymatic function are the same (with perhaps a small change in the substrate specificity), homology studies are then undertaken using MODELLER; which takes the secondary structure of MCR and threads the AMACR sequence through it. This then automatically builds the tertiary structure. Approximately 50 models are generated and the lowest energy model is used. As a caveat however, the model at loop regions can sometimes be mis-represented. However, since the catalytic site is in a groove, rather than at a loop region, this is not too important. The AMACR sequence has an additional 30 amino acids at the C-terminal, so this cannot be modelled in (Fig 1.20)

AMACR **MCR**

Fig 1.20. MCR and AMACR model comparison with the catalytic residues histidine and aspartate in shown as spheres.

Three completely conserved residues appear to be suitably positioned to act as catalytic residues in AMACR, namely His122, Asp123, and Asp152. The CoA stacking residue Arg81 is also conserved. Lys 58 is conserved and Arg 91 of MCR is replaced by Lys87, which is salt bridged to the 3'-phosphate moiety. The methionines in the hydrophobic pocket of MCR are replaced by hydrophobic residues of phenylalanine, leucine and tryptophan. All in all, the high homology between prokaryotic and eukaryotic isoforms of AMACR suggests that their function, substrate specificity and catalytic mode of action are likely to be very well conserved.

1.7 Fatty Acid Metabolism as a Therapeutic Target.

One of the consistently observable features of PCa is the upregulation of fatty acid metabolism to meet the bioenergetic requirement for rapid cell proliferation. Several enzymes have been identified as being overexpressed. *Fatty acid synthase* (FAS) is considered to be a possible metabolic oncogene in prostate cancer[108] and is found to be overexpressed at both protein and mRNA levels.[109, 110] Inhibiting FAS with Cerulenin and C75 (established FAS inhibitors) has demonstrated significant antitumor activity. Both Cerulenin and C75 have however been shown to cause profound effects on food intake and bodyweight in mice that could limit the development of FAS inhibition as a therapeutic strategy.[111] To date, the most potent inhibitor of FAS is GSK837149A, developed by GSK but biochemical studies have shown it to have poor cell permeability.

Studies on the upregulation of the β-oxidation pathway by Zha *et al.*,[112] have shown an increased expression of mRNA, protein and accompanied enzymatic activity of MFE-2. Furthermore, ACOX3, which is expressed at extremely low levels in other human organs studied, including the liver, may contribute significantly to peroxisomal branched chain fatty-acid β-oxidation in human prostate tissue, with possible greater significance in PCa.[113]

Using small interference RNA (siRNA) to impair AMACR expression significantly impaired proliferation of the androgen-responsive PCa cell line LAPC-4 as an *in vitro* model. The mechanism of this growth inhibition was found to be independent of androgen, indicating that AMACR is essential for optimal growth of PCa cells.[114] More recent studies on AMACR's involvement in conversion of prostate cancer from hormone independence to dependency have shown that by inhibiting AMACR expression in the androgen independent cell line C4-2, there is an increased expression of AR. Incubating the androgen dependent cell line LNCaP with an androgen depleted serum demonstrated significant decrease of cell viability, whereas in the same serum, the C4-2 cells grew normally. However, when incubated in an androgen depleted serum treated with AMACR siRNA, the C4-2 cell line showed a significant decrease in cell viability.[115] These results indicate that the expression of AR due to AMACR inhibition may induce characteristic conversion of prostate cancer cells from a hormone independent to hormone dependent state. This is potentially hugely advantageous when dealing with hormone refractory PCa.

There are two thoughts in regards to AMACR's role in the development of prostate cancer. The first being it helps to provide metabolic support necessary for tumour growth and the second theory is that increased peroxisomal AMACR activity is responsible for increasing peroxisomal β-oxidation, resulting in increased cellular peroxide production *via* the ACOX enzymes. This increased production of cellular peroxide, without a compensatory increase in catalase expression, results in a more oxidative environment in prostate cancer cells, promoting DNA damage and increasing the risk of prostate cancer from high-grade prostatic intraepithelial neoplasia.

Using AMACR as a therapeutic target for prostate cancer seems an attractive strategy. Targeting other enzymes that are upregulated in the β-oxidation pathway (MFE-1/2 ACOX etc.), may lead to severe side effects and additional toxicities (as described in Section 1.4.1) whereas inhibiting the functionality of AMACR leads to rather milder complaints that can be

alleviated with a controlled diet. Additionally, it has been suggested that AMACR could be targeted as a promising avenue of treatment for hormone independent prostate cancer for which at present there are no effective treatments.

Upon designing inhibitors of AMACR, one should consider enzyme specificity. Around 4% of all cellular enzymes are CoA utilising. The CoA moiety is frequently a prerequisite for the binding of substrates to enzymes, with many possible hydrophilic interactions. Making alterations to this fragment but still maintaining enzyme affinity is thus difficult. However, there is scope to increase enzyme specificity with regards the acyl region of the CoA thioester. Many CoA utilising enzymes use shorter groups extending from the thioester. Metabolism of longer acyl coenzyme A thioesters is normally restricted to the β-oxidation pathway. Incorporating compounds that have an α-methyl group may increase specificity further towards the specific set of α–methyl utilising enzymes.

1.8 Established and proposed inhibitors/substrates of AMACR

1.8.1 Probing the inhibition of AMACR

Initial inhibition studies of rat AMACR were carried out by Schimtz *et al.*[82] A range of reagents were incubated with the enzyme and the rate of which [2-^{3}H]-pristanoyl-CoA exchanged with the solvent was monitored. It was noted that there was strong inhibition of the enzymatic activity by thiol directed reagents such as Hg^{2+} or NBS, which suggest the presence and availability of a cysteine residue. The enzyme was also largely inactivated by diisopropylphosphofluoridate, which is generally known as a specific and potent inhibitor of serine. However, these specific amino acid binders do not necessarily mean that they are involved in the catalytic mechanism of the enzyme. Of the branched-chain fatty acyl-CoAs tested, only 2-methyloctanoyl-CoA and 2-methylmyristoyl-CoA had an inhibitory effect. Neither the short chain 2-methylpropionyl-CoA, 2-methylbutyryl-CoA nor the phytanic acid (a 3-methyl branched fatty acid derivative) had any effect. The α,β,unsaturated compound, 2-methylmyrist-2-enoyl-CoA, also did not inhibit the reaction, indicating a clear substrate specificity of the enzyme for saturated medium and long-chain α-methyl branched fatty acids. Since these initial studies only Carnell *et al.,* have performed further inhibition studies on AMACR.[116]

1.8.2 Designed suicide inhibitors

Prior to the publication of the bacterial crystal structure[61], a series of substrate analogues were synthesised as mechanism-based inhibitors containing one or more fluorine atoms in a β-position to the CoA thioester[116].

Alanine racemase (a PLP-dependent (**38**) bacterial enzyme that catalyses the racemisation of alanine, providing *D*-alanine for cell wall synthesis[117]) is one of the most studied racemases and achieves chiral inversion by deprotonation and reprotonation by a catalytic acid/base pair. Inhibition of alanine racemase with β,β,β-trifluoroalanine (**39**) has been studied previously by Walsh *et al.*[118] It was found that incubation of β,β,β-trifluoroalanine led to irreversible inhibition where the enolisation process (**40**) leads to the formation of an α,β-unsaturated intermediate (**41**) which can undergo nucleophilic attack by a basic amino acid residue within the active site. (Fig 1.21)

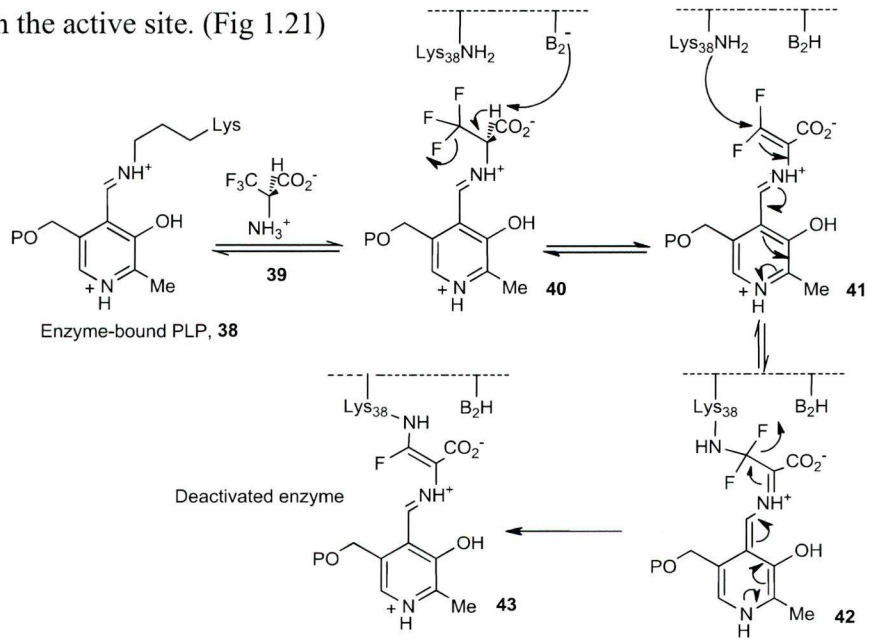


Fig 1.21. A Schematic representation of the irreversible inhibition of alanine racemise by β,β,β-trifluoroalanine. B_2 represents Tyrosine 265.[118]

It was thus envisaged that this type of inhibition could be replicated with AMACR; whereby elimination of a fluoride from the proposed enolate (**45**) by an E1cB mechanism within the active site would generate an α,β-unsaturated thioester (**46**) that may then undergo attack by an active site base or nucleophile to give the inactivated enzyme (**48**) (Fig 1.22)

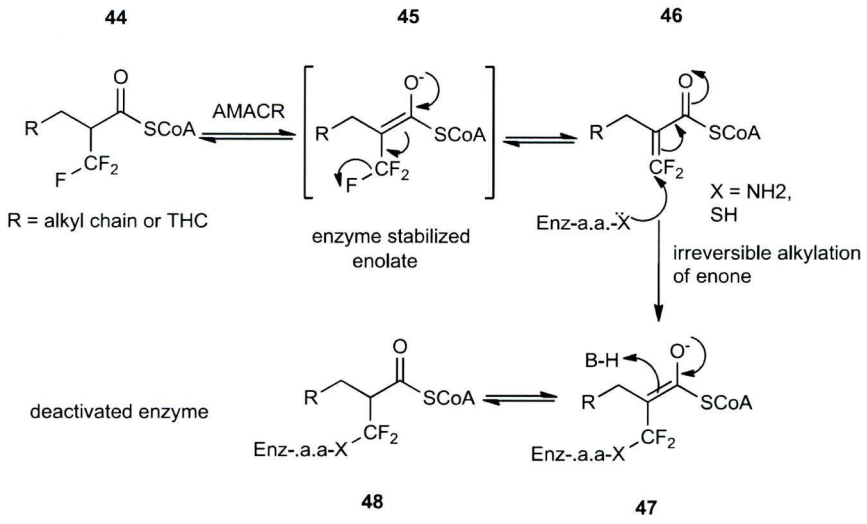


Fig 1.22. A proposed mechanism of inhibition of AMACR by β-fluorinated analogues of natural substrates.

However it was found that these postulated irrevsible inhibitors inhibited the enzyme competitively (Fig. 1.23).[116] The most successful of these inhibitors is 2-trifluoromethyltetradecanoyl-CoA (**49**). This has a 150 fold increase in activity compared to its non fluorinated analogue 2-methyltetradecanoyl-CoA (**53**) with a K_i of 0.9μM (based on two different assays). To date, this is the most potent inhibitor of rat AMACR. Another interesting point to make with regards to these results is the difference in K_i value between (*S*) and (*R*)-ibuprofenoyl-CoA (**22a** and **22b**). (*R*)-ibuprofenoyl-CoA (**22b**) has a lower K_i value then its (S) counterpart (**22a**). This is something that should be considered when designing potential inhibitors.

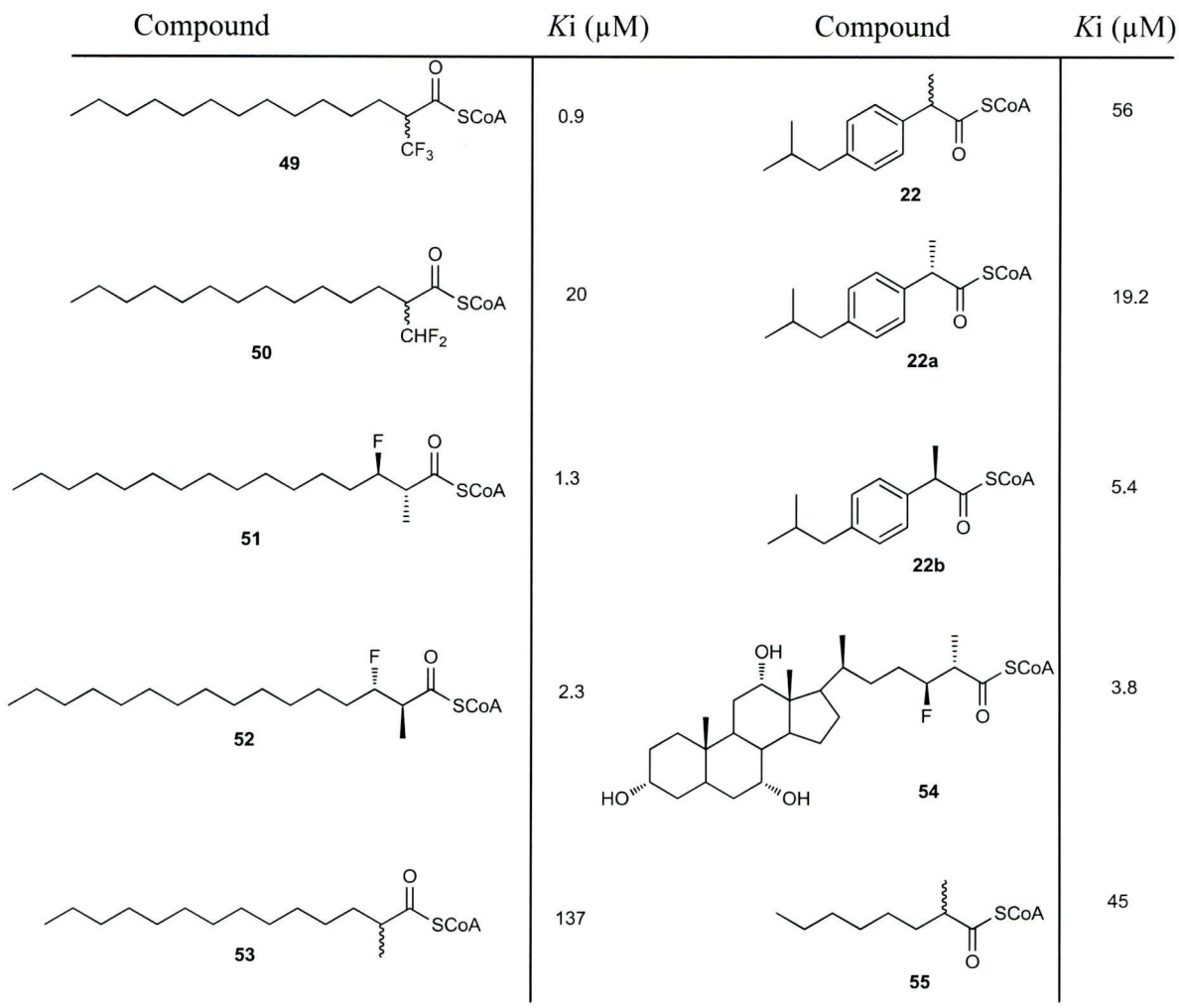


Fig 1.23. A range of competitive inhibitors of AMACR and their *K*i values.

From the crystal structure of MCR and mechanistic studies of AMACR, we can hypothesise the rationale of these K_i's. Since the trifluoro group is electronegative, this would lower the p*K*a of the α-proton, facilitating enolisation. The fact that all mono β-fluorinated compounds (with the exception of compound **50**) have similar K_i values of 1.3-3.8μM is also an additional verification of the proposed mechanism of inhibition. Moreover, the fluorinated enolate intermediate, may bind more strongly at the active site

However, there is no direct evidence that the competitive inhibition is due to the lowering of the p*K*a of the α-proton, and so one must also consider that the inhibition may be due to an increased binding affinity of the substrate. This is one of the questions that we wish to answer by synthesising non-enolisable α-fluoro analogues and comparing their relative *K*i's.

1.8.3 Proposed mechanism-based inhibitors

We have designed a series of AMACR inhibitors that have similar properties to those that have already been synthesised and tested. These inhibitors are designed to probe three aspects of the substrate enzyme interaction; the first being the binding affinity between the acyl group and the hydrophobic pocket, the second to probe the methyl binding pocket, and the third, the catalytic mechanism (Fig 1.24).

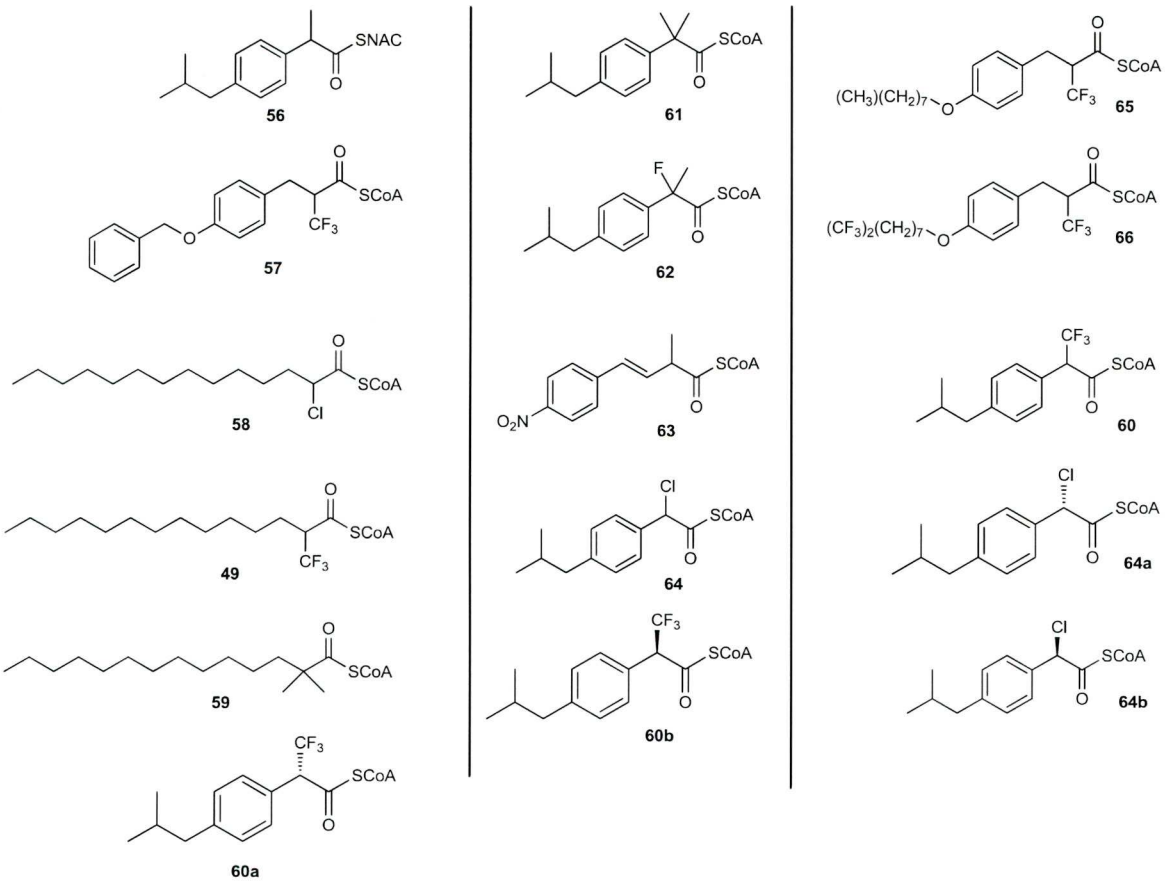


Fig 1.24. A range of proposed compounds to probe inhibition and mechanism of AMACR.

We have designed several substrates that will aid us in developing an assay in which to test our potential inhibitors. Two LC based assays were created along with developing a fast, reliable chromogenic assay. It was proposed that we could monitor the rate of the racemisation by following the rate of formation of (*R*)-ibuprofenoyl-CoA (**22b**) from the enantiomerically pure (*S*)-ibuprofenoyl-CoA (**22a**). This was to be done using chiral RP-HPLC to measure the relative increase of (*R*)-ibuprofenoyl-CoA (**22b**) formed. The second LC based assay involves the use of LCMS. We would synthesise a racemic mixture of α-

deutero-ibuprofenoyl-CoA (**67**) and measure the formation of the ibuprofenoyl-CoA (**22**) by injecting aliquot portions of the reaction mixture onto an LCMS system equipped with an RP column (Fig 1.25).

Fig 1.25. α–deuterio-Ibuprofenoyl-CoA and (*S*)-Ibuprofenyol-CoA as substrates for an LC based assay.

The chromogenic substrates (**68/69/72**) were designed to form the chromophore (**71/74**) through enzymatic abstraction of the α-proton of both chromogens to form the enzyme-stabilised intermediates (**70/73**) followed by an E1cB elimination step to generate a conjugated chromophore (**71/74**) (Fig 1.26).

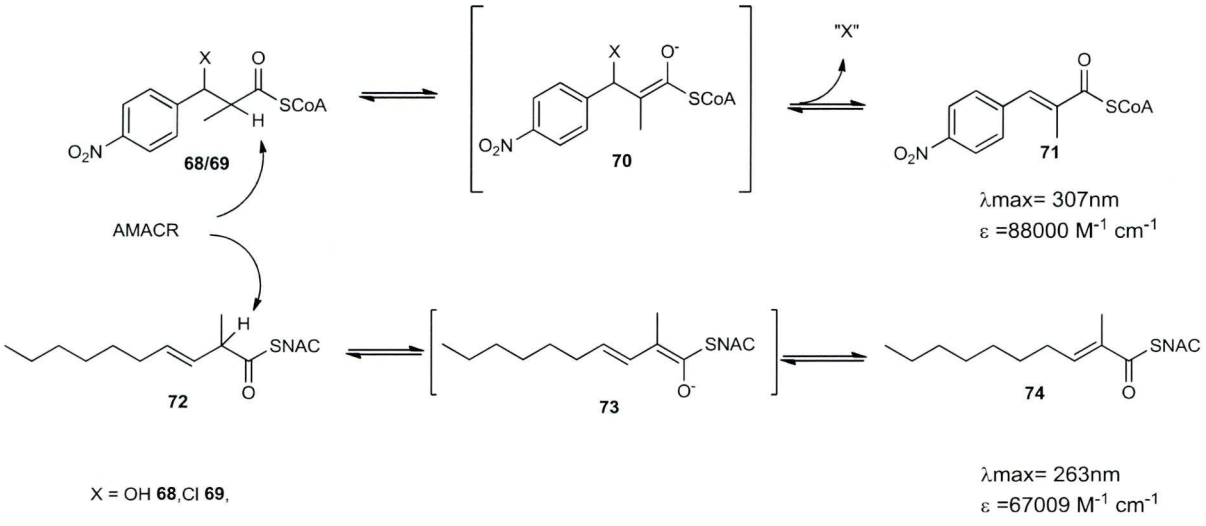


Fig 1.26. A Schematic representation of the proposed formation of a UV detectable chromophore through AMACR promoted E1cB elimination.

We propose the synthesis of two different thioesters as potential substrates for our chromogenic assay (fig 1.27). The first being the conventional CoA thioester and the second being an *N*-acetylcysteamine thioester. The reason for the differences in the thioester moiety is because upon formation of the aliphatic chromophore (**74**), a UV maxima of 263nm is produced.[119, 120] Adenine (which is an intrinsic part of the CoA molecule) has an extinction

coefficient of 15800 at a wavelength of 260nm.[121] This could interfere with any possible measurements of the formation of the aliphatic chromophore (**74**). The *p*-nitro chromophore (**71**) has a UV maxiumum at 307nm[122] which should not overlap with the CoA UV maxima. The second rationale for using N-acetylcysteamine is that it has recently been reported that a number of fatty acyl-CoA ligases (FACL's) display remarkable tolerance when the CoA group is replaced by *N*-acetylcysteamide (NAC).[123] It is known that FACL's contain overlapping substrate specificities,[124] which has also been proposed for AMACR and related β-oxidation enzymes. Furthermore, it was found that varying the chain length did not affect the formation of fatty acyl-CoA thioesters. These properties draw parallels with AMACR, indicating that NAC thioesters may indeed be tolerated as an alternative to CoA-thioesters.

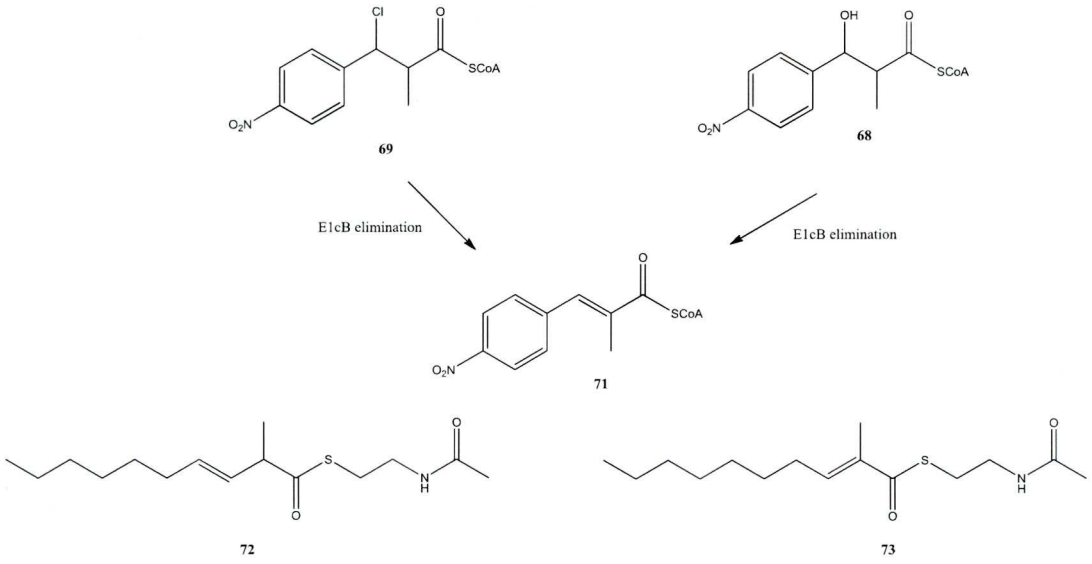


Fig 1.27. A range of potential chromogenic substrates and their respective chromophores.

CHAPTER 2
SYNTHESIS OF INHIBITORS

2.1 Project Aims

Competitive inhibition studies carried out by Carnell *et al.*,[116] in addition to the structural and mechanistic studies carried out by Wierenga *et al.*,[61] and Lloyd *et al.*,[85, 102] indicate that the α-methyl and CoA thioester moieties in the substrate are crucial for binding with the active site. These findings also suggest the presence of a large hydrophobic region within the catalytic site, which allows for structural diversity in potential inhibitors and substrates. We have designed a series of inhibitors with a high degree of structural similarity to the natural substrates and to previously recognised inhibitors of AMACR. This chapter discusses strategies employed and the rationale behind the synthesis of these potential inhibitors.

2.2 Modified synthesis of α-trifluoromethyltetradecanoyl-CoA (**49**)

First we shall discuss the re-synthesis of the most potent inhibitor to date, α-trifluoromethyltetradecanoyl-CoA (**49**). This was previously synthesised by Ian Hale *et al.*,[125] by performing a Wittig reaction between the dodecyl triphenylphosphonium bromide salt (**75**) and the commercially available trifluoromethyl pyruvate to yield a 1:1 mixture of *E* and *Z* stereoisomers of the enolate (**76**) (Fig 2.01). Conventionally, we would expect the *Z*-isomer to predominate due to the unstabilised nature of the ylide. However, this ratio has previously been observed by Palecek and co-workers in their preparation of trifluoromethyl α,β-unsaturated esters[126] using the same Wittig reaction conditions. This was followed by hydrogenation (using 10% Pd-C in methanol) and basic hydrolysis to give the free acid (**79**). Attempts at using ethyl chloroformate and triethylamine to form a mixed anhydride proved unsuccessful in coupling to coenzyme A. However, the coupling of the free acid to coenzyme A using CDI proved a successful alternative (Fig 2.01).

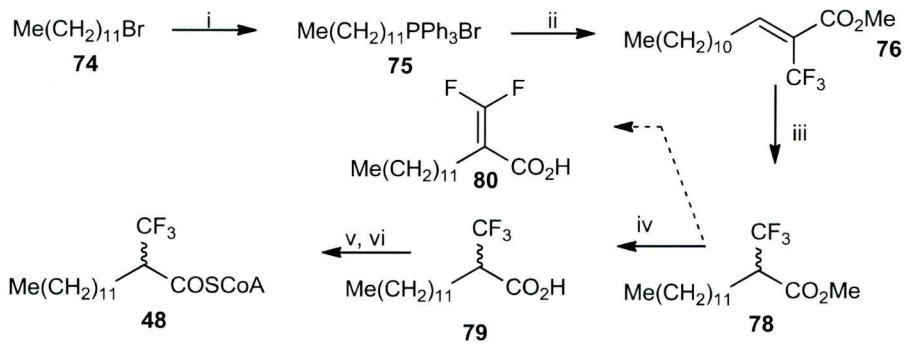


Fig 2.01 - *Reagents and conditions*: i, PPh_3, toluene, Δ, 48h, 98%; ii, *n*BuLi, $F_3CC(O)CO_2Me$, THF, -78^{0}C, 3h, 54%; iii, H_2/Pd/C, MeOH, rt, 24h, 94%; iv, 5*N* NaOH, THF, MeOH, rt, 24h, 97%; v, CDI, THF, rt, 2h; vi, CoA-SH, $(Li^+)_3$, H_2O/THF, rt, 24h.

Attempts at repeating this work were troublesome. The synthesis of 2-trifluoromethyltetradecanoic methyl ester (**78**) was achieved using the previously established route. Use of 5*N* NaOH in a mixture of THF and methanol to hydrolyse the methyl ester did not give the desired free acid. Instead, it was noted that HF was removed by a probable β-elimination pathway (**80**) (Fig 2.01 – dashed). There is only one literature precedent which describes a similar phenonomen with an aliphatic α-trihalomethyl ester. The β-elimination process occurred with the use of the surprisingly mild potassium carbonate base.[127] A range of hydrolytic conditions were subsequently tested in order to obtain the desired acid. Using a lower concentration of base yielded the same undesired product, as did using less equivalents and changing the base from sodium hydroxide to potassium hydroxide or lithium hydroxide. Similarly, attempts at hydrolysing the methyl ester in its unsaturated form (**76**) did not yield the desired acid, but instead also resulted in elimination of fluorine. Refluxing the methyl ester with barium hydroxide surprisingly resulted in no reaction at all and the starting material was fully recovered. Attempts at using acidic methods also proved fruitless. Boiling the methyl ester in concentrated sulphuric acid did not yield the acid, nor did the use of concentrated hydrochloric acid. In both cases no hydrolysis was achieved. It has been reported that α-trifluoromethyl aliphatic esters can be enzymatically hydrolysed by Lipase PS.[128] However, this is not the case for this substrate. A range of other lipases were also screened, giving no product and full recovery of the starting material.

A longer alternative route was thus devised involving reduction of the methyl ester to the corresponding alcohol and then oxidation to the free acid. Reduction of the ester using lithium aluminium hydride to give the alcohol worked well in good yield. However,

oxidation was problematic. Oxidation using a single step procedure such as Jones oxidation gave a range of products by TLC. Milder stepwise oxidation procedures such as the use of PCC or Swern conditions to yield the desired aldehyde were prohibitively low yielding.

Another method of de-esterification for alkyl esters is to reflux the ester with lithium iodide in wet DMF. Traditionally, these reagents and conditions are used to convert β-ketoesters (**81**) to ketones (**84**) through decarboxylation (**82-83**). This is known as Krapcho decarboxylation (Fig 2.02).

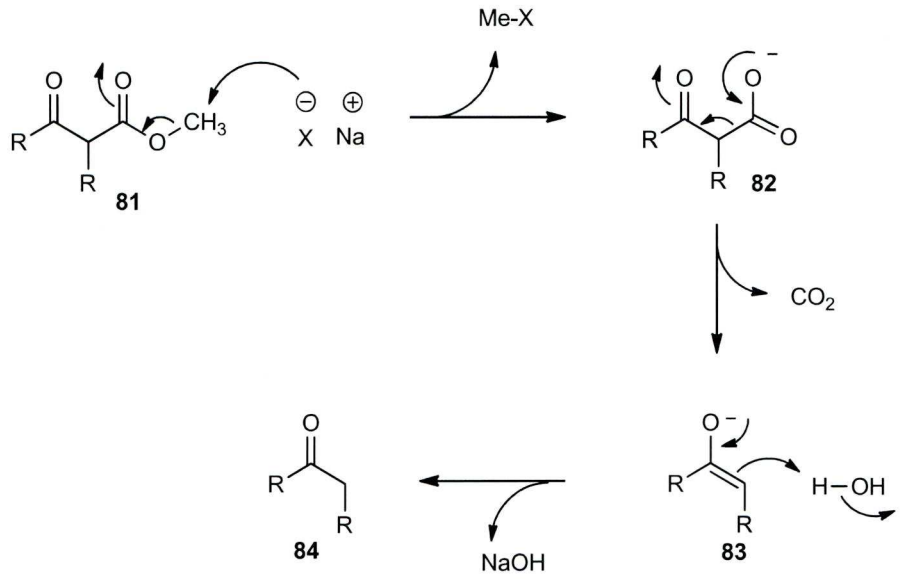


Fig 2.02. Krapcho decarboxylation.

Using the Krapcho decarboxylation conditions for de-esterification purposes has been known for the last half a century[129] but is employed infrequently. It is particularly useful for methyl esters since the de-esterification reaction is an S_N2 process. We found that refluxing compound (**78**) with lithium iodide in wet DMF between 8 hours and overnight furnished the desired free acid (**79**) in an excellent yield. The CoA ester was made by activating the free acid with CDI (Fig 2.03). This was then purified by reverse phase HPLC. A description of the development of purification of CoA thioesters by HPLC can be read in Section 3.2, exemplified by Ibuprofenoyl-CoA (**22**).

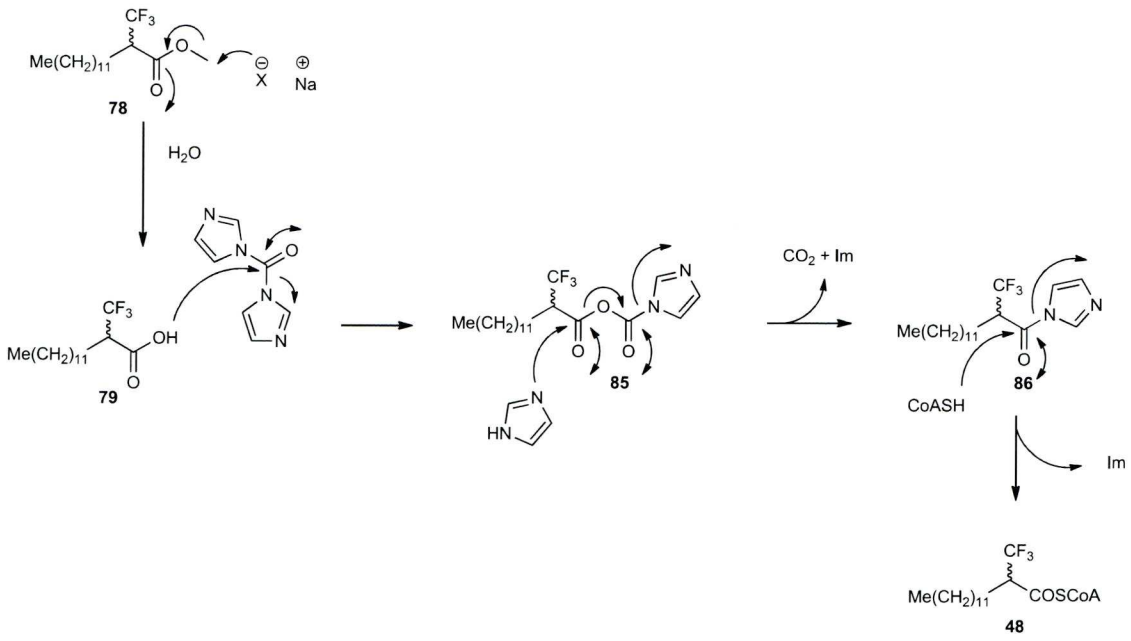


Fig 2.03. Krapcho de-esterification followed by activation of the free acid with 1,1-carbonyldiimidazole and the subsequent thioesterification.

2.3 Incorporation of an α-dimethyl group

It is well established that AMACR accepts both enantiomers of its substrates, with little preference for one over the other.[102] By synthesising dimethylpentadecanoyl-CoA (**59**) we wish to probe two aspects of the inhibitory binding. Firstly, having both 'orientations' of the methyl group present in a single molecule might increase the binding affinity of the substrate to the enzyme and secondly, without the α-hydrogen, the compound is unable to enolise. We can thus study whether it is the enolate or the ester that is the real inhibitor. Treatment of isobutyric acid (**87**) with sodium hydride and diisopropylamine gave an enolate dianion which then reacted with 1-bromododecane to yield 2,2-dimethylpentadecanoic acid (**88**) (Fig 2.04). This can then be thioesterified with CoASH as described in Section 2.2.

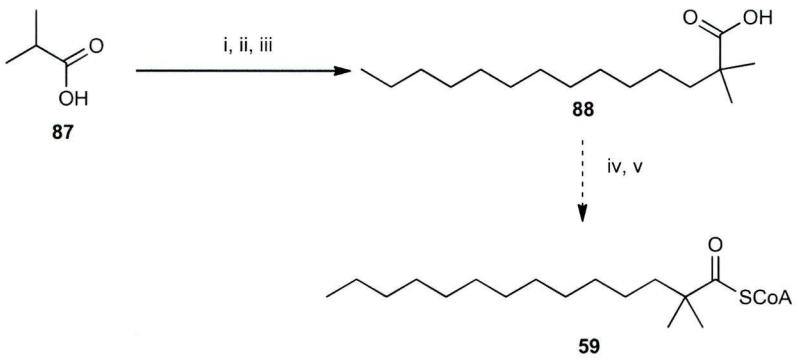


Fig 2.04 *Reagents and conditions*: i, NaH, DIPA, THF, rt, 20mins; ii, $CH_3(CH_2)_{11}Br$, 0°C, 60mins, then rt ON; iii, aq HCl, 0°C, 5mins, 58%. iv, CDI, THF, rt, 2h; v, CoA-SH, $(Li^+)_3$ THF/H_2O, rt, 24h.

2.4 Incorporation of an α–chloro moiety (**58**).

The natural substrates of AMACR previously discussed contain an α-methyl moiety. Chlorine is known as an isosteric replacement of a methyl group.[130] It can also isosterically replace a CF_3 group or CN group. Replacing the α-methyl group with chlorine is a compelling choice for a potential inhibitor as it not only has a similar size to that of the α-methyl group, but chlorine is electronegative, which lowers the p*K*a of the α-proton, thus possibly inhibiting in a similar manner to that of α-trifluoromethyltetradecanoyl-CoA (**49**). The synthesis of the free acid was achieved by following a protocol found in the textbook by Vogel.[131]

Treatment of myristic acid (**89**) with thionyl chloride gave tetradecanoyl chloride (**90**) which was converted to its α-chloro analogue (2-chlorotetradecanoyl chloride (**91**)) by subsequent treatment with 2 mole equivalents of *N*-chlorosuccinamide and concentrated hydrochloric acid. The fractionally distilled 2-chlorotetradecanoyl chloride (**91**) was then subject to hydrolysis using mildly acidic conditions to yield 2-chlorotetradecanoic acid[132] (**92**). This could then be subjected to the previously stated CoA thioesterifcation procedure (Fig 2.05). This was not done since it was thought that the α–chloro acid would be more stable in storage.

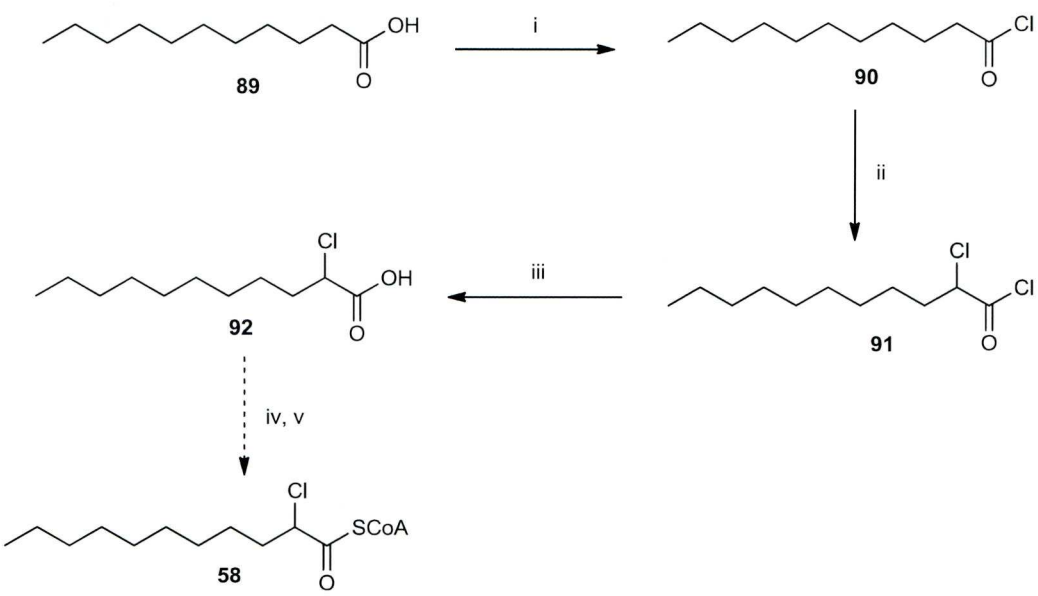


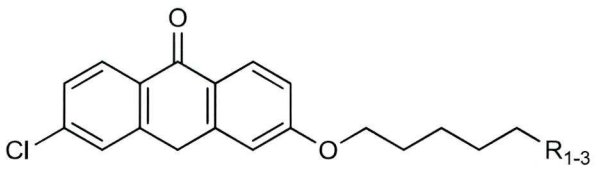
Fig 2.05 *Reagents and conditions*: i, $SOCl_2$, Δ, 30 mins; ii, $SOCl_2$, NCS, HCl, 80°C, 1.25h; iii, aq HCl, rt, 5 mins, 54%. iv, CDI, THF, rt, 2h; v, CoA-SH, $(Li^+)_3$ THF/H_2O, rt, 24h.

2.5 Synthesis of aryl ether derivatives.

2.5.1 Rationale

It is well understood that fluorine exerts some unique properties on biological molecules.[133] The C-F bond is notoriously strong, with a bond strength in the region of 115 kcal mol^{-1}, in comparison to the C-H bond, which has an average bond strength of 104 kcal mol^{-1}. [134] This means that introduction of a C-F bond can be used to prevent metabolism.

Studies on anti-malarial Acridones by Winter *et al.,* have shown some interesting results. Acridones were designed to inhibit the *Plasmodium* cytochrome bc1 complex.[135] This enzyme has a relatively large hydrophobic pocket and it was found that the potency of the acridones were reliant on the variation of the aliphatic region. The general relationship was that the more hydrophobic the aliphatic tail, the better the IC_{50} of the acridone (**93-95**) (Fig 2.06). Presumably, this relationship occurs by an increased affinity to the active site.



R_1 = Cl, IC_{50} = 12nM, **93**
R_2 = CF3, IC_{50} = 0.3nM, **94**
R_3 = $C(CF_3)_2F$ IC_{50} = 1pm, **95**

Fig 2.06 Various acridone analogues that demonstrate the influence of fluorine atoms on antimalarial potency.

The same general argument could be proposed for AMACR. As with *Plasmodium* cytochrome bc1 complex, AMACR has a large hydrophobic pocket that consists of methionines, leucines, tyrosines and phenylalanines. Designing compounds that incorporate a terminal polyfluoroaliphatic moiety, accompanied by the α-trifluoromethyl group (Fig 2.07), which had already been established to exert beneficial effect on K_i compared to the natural substrates, may decrease the K_i further. It was envisaged that incorporation of an aromatic group between the α-methyl functionality and the aliphatic perfluoro moiety would give the potential inhibitor structural rigidity. Other derivatives such as compound (**66**) (the polyfluorinated tail replaced by a hydrocarbon) and compound (**96**) (the α-trifluoromethyl group is replaced with a methyl group) (Fig 2.07) are also useful probes. If the two α-trifluoromethyl compounds have similar *Ki* values, but the α-methyl derivative with the fluorinated tail showed a relatively poor *Ki* in comparison, this would indicate that the binding affinity of the inhibitor is not due to the hydrophobic tail but would indicate the importance of the α-trifluoromethyl group - either by the lowering of p*K*a of the α-proton or the general affinity of the α-trifluoro group to the methyl pocket.

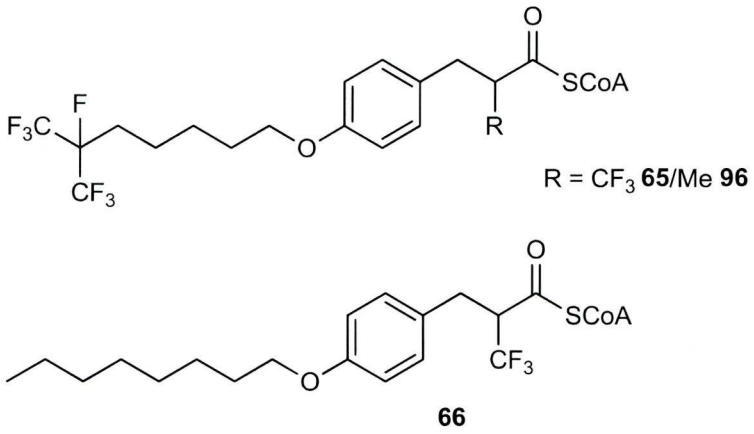


Fig 2.07 Variations of the aryl ether inhibitors.

The optimal length of the aliphatic region in inhibitors (**65**)/(**66**)/(**96**) was calculated using Spartan. From previous reports, it was shown that the length of the aliphatic moiety extending from the thioester must be at least 8 carbon atoms long. Using our most potent inhibitor (**49**) as a model (14 carbons), the length was matched with our aryl ether compound(s). It was found that an additional 8 carbons extending from the ether linkage fitted best. 1,1,1-Trifluoro-8-halo octanes are not commercially available. However, 1-bromo octanoic acid is. Work by Lal *et al.,* suggested that free aliphatic acids can be converted into trifluoro groups by reacting the acid in neat deoxofluor[136]. Deoxofluor is known to be a mild form of DAST (diethylaminosulfur trifluoride) and a common commercial source of nucleophilic fluorine. Repeating this reaction was challenging. This was due to the difficulty in isolating the product. A range of compounds were formed (noted by TLC). Furthermore it was difficult to quantify the extent of the reaction and to determine whether the TLC spots were intermediates of the final compound or side products. To partially circumvent this problem it was decided to work with the commercially available 1,1,1,3,3,3-trifluoro-2-fluoro-6-bromohexane. Although this is two carbons shorter, it adheres to the minimum of 8 carbons rule.

2.5.2 Heck coupling with trifluoromethyl acrylic acid

Our original approach for synthesis of these compounds was to perform a Heck coupling reaction between a 1-halo-4-alkoxy benzene (**100**) and a commercially available trifluoromethyl acrylic acid (Fig 2.09). This would then undergo hydrogenation to reduce the double bond followed by coupling to coenzyme A. We initially tested this novel Heck reaction with bromobenzene (**97**) and trifluoromethyl acrylic acid (**98**) using palladium

acetate, triphenylphosphine and two equivalents of caesium carbonate. Although the reaction worked, the yield was poor (21%, fig 2.08). Attempting this on a *para* ether compound such as *para* bromo anisole did not yield the desired compound, even if the base or ligand was varied (Cs_2CO_3 and NEt_3 as bases, PPh_3 and $P(Otol)_3$ as ligands). Using these conditions caused polymerisation of trifluoromethyl acrylic acid (noted by NMR and mass spectrometry). We attempted to overcome this problem by the addition of hydroquinone, which can help suppress polymerisation. However, this was unfortunately ineffective. Sidestepping polymerisation by protecting the carboxylic acid moiety, we synthesised benzyl 2-(trifluoromethyl)acrylate (**102**) by refluxing trifluromethyl acrylic acid (**98**) in benzyl alcohol with a catalytic amount of H_2SO_4. The Heck coupling was repeated but still no desired product was isolated (Fig 2.08). Changing the X group from bromo to iodo (which facilitates the oxidative addition) still yielded no product. As the *para* methoxy (or analogue) is electron donating, a plausible explanation may be due to an increased stability of the C-X bond. This decelerates the oxidative insertion of Pd, rendering trifluoromethyl acrylic acid (or analogue) more susceptible to undergo polymerisation. Moreover, there is only one reference of a Heck coupling reaction with an α-trifluoromethyl moiety (relative to the least hindered side of the double bond). Even then, this was restricted to 3,3,3-trifluoro-2-methylprop-1-ene[137]; otherwise, it is unprecedented. Although theoretically possible, it is practically very challenging. Therefore a new route was devised.

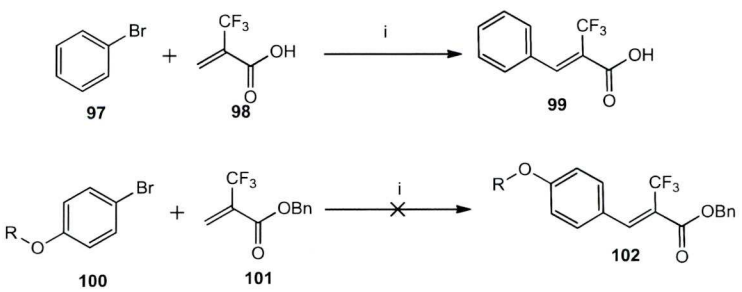


Fig 2.08 Heck coupling reactions of trifluoromethyl acrylic acid with a variety of bromo aromatic compounds. *Reagents and conditions*: i, PPh_3, $Pd(OAc)_2$, Cs_2CO_3, DMF, 150°C, O.N 21%.

2.5.3 Successful synthesis of aryl ether derivatives

Changing strategies involved creating an ether linkage between *para* hydroxy benzyl alcohol and the desired aliphatic chain. The benzyl hydroxy group was replaced by iodine, which was subsequently converted to a phosphonium salt, suitable for a Wittig reaction with trifluoromethyl pyruvate. The resulting double bond was then reduced, followed by de-esterification and finally CoA thioesterification (Fig 2.09).

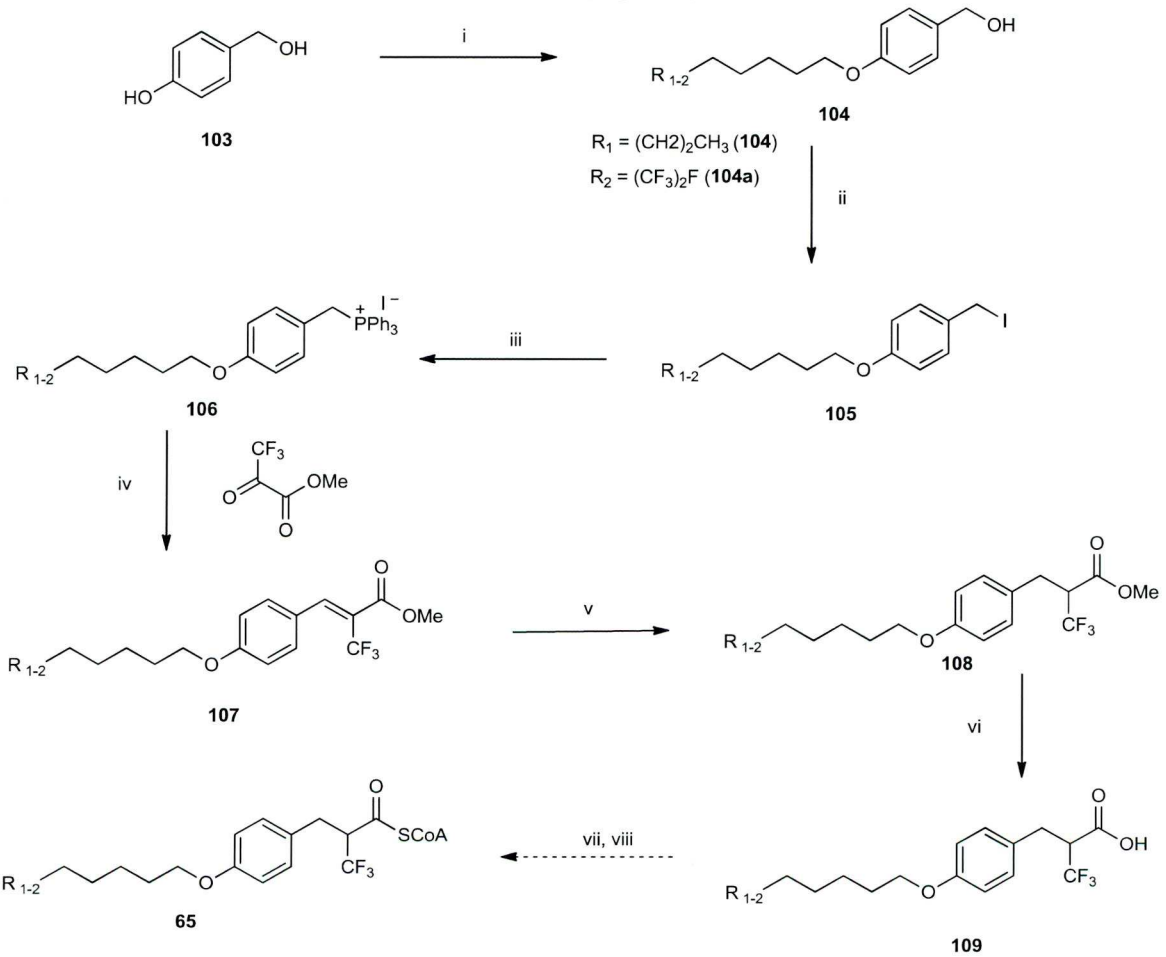


Fig 2.09. *Reagents and conditions* for R_1 = : i, K_2CO_3, $CH_3(CH_2)_7Br$, Me_2CO, 60°C, 4h, 83%; ii, PPh_3, Imid, I_2, DCM, 25°C, 30 mins 98%; iii, PPh_3, Tol, Δ, 3h 87%; iv, n-BuLi, $CF_3C(O)C(O)OCH_3$, THF, -78°C, 2h 34%; v, Pt-C, H_2, MeOH, 800psi 89%; vi, LiI, DMF 180°C, 8h, 70%; vii, CDI, THF, rt, 2h; viii, CoA-SH, $(Li^+)_3$ THF/H_2O, rt, 24h.

Reacting the aliphatic chain with *para* hydroxyl benzyl alcohol (**103**) in the presence of potassium carbonate in acetone[138] yielded the desired ether (**104**) in good yield. It does not create an ether linkage at the aliphatic alcohol as the p*K*a of the aromatic hydroxyl is lower

(p*K*a of an aromatic OH ≈10 as compared to the p*K*a of aliphatic OH≈16). Next, the hydroxy group was displaced with a halide. This can be achieved in a number of ways including the use of thionyl chloride/oxalyl chloride, the use of phosphorus tribromide, or the use of iodide. The iodo derivative was chosen in this instance. The R-I bond is weakest out of the available halogens, thus facilitating the following Wittig reaction. To perform the iodoination, it was decided to use triphenylphosphine, iodine and imidazole[139] (Fig 2.10). The reaction time was less than an hour and a quantitative yield was obtained. It should be noted that mass spectra of the iodo compounds were not obtained due to α-cleavage.

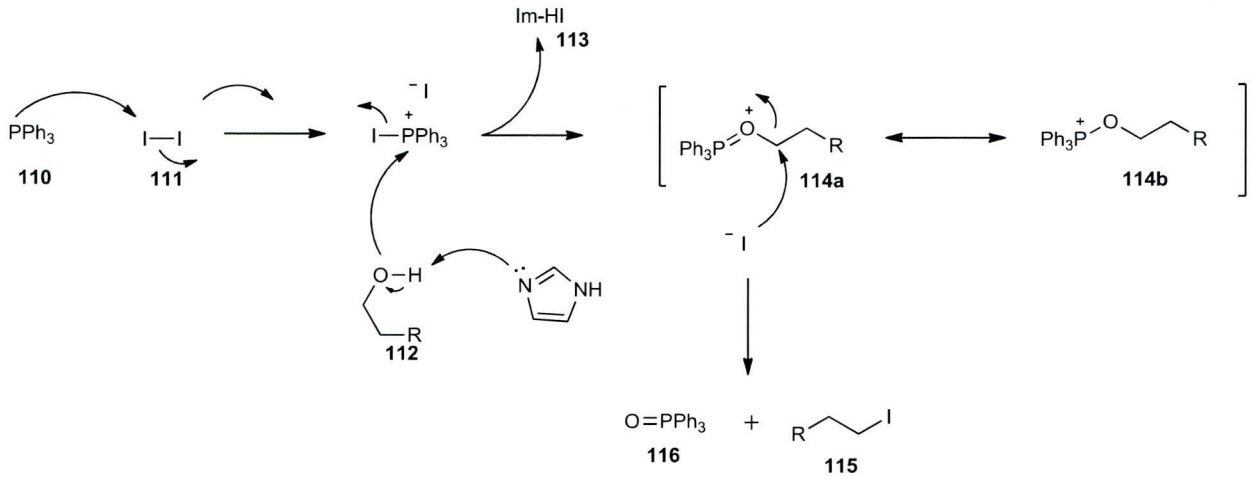


Fig 2.10. Mechanism of halogen displacement.

After formation of the corresponding phosphonium salt[140](**106**), a Wittig reaction was performed with trifluoro pyruvate. This gave (**107**) in a good yield with reproducible stereoisomeric ratios of 1:1. Initial attempts at reducing the double bond using H_2/Pd-C at atmospheric pressure in methanol yielded no product and the starting material was fully recovered. Increasing pressure to 800 psi gave a similar outcome. Changing the catalyst to Pt-C at 800 psi reduced the double bond successfully to yield (**108**). The low reactivity can be attributed to the fact that the neighbouring trifluoro group deactivates the double bond. Using Pt rather the Pd overcame this problem. The aryl ether (**108**) was then de-esterified under Krapcho conditions to yield the desired acid (**109**).

2.5.4 Alternative synthetic strategy

As an alternative more versatile synthetic route to this series of compounds, another strategy was also employed. A benzyl ether linkage between the 4-hydroxy benzyl alcohol (**117**) and benzyl bromide was created[141] (**118**). Routine procedures to form the unsaturated methyl ester were then executed. It was envisaged that the double bond and benzyl group would be reduced and removed respectively by using Pt-C in methanol in order to then introduce a phenolic alkyl group of our choosing. However, use of Pt-C at 800 psi overnight resulted in only reduction of the double bond (**122**) (Fig 2.11). The fact the Pt-C did not remove the benzyl group could be considered serendipitous. Although another reagent (Pd-C) is needed to gain access to a range of aliphatic aryl ethers, we now have access to compound (**56**), which will add to our arsenal of hydrophobic pocket probes.

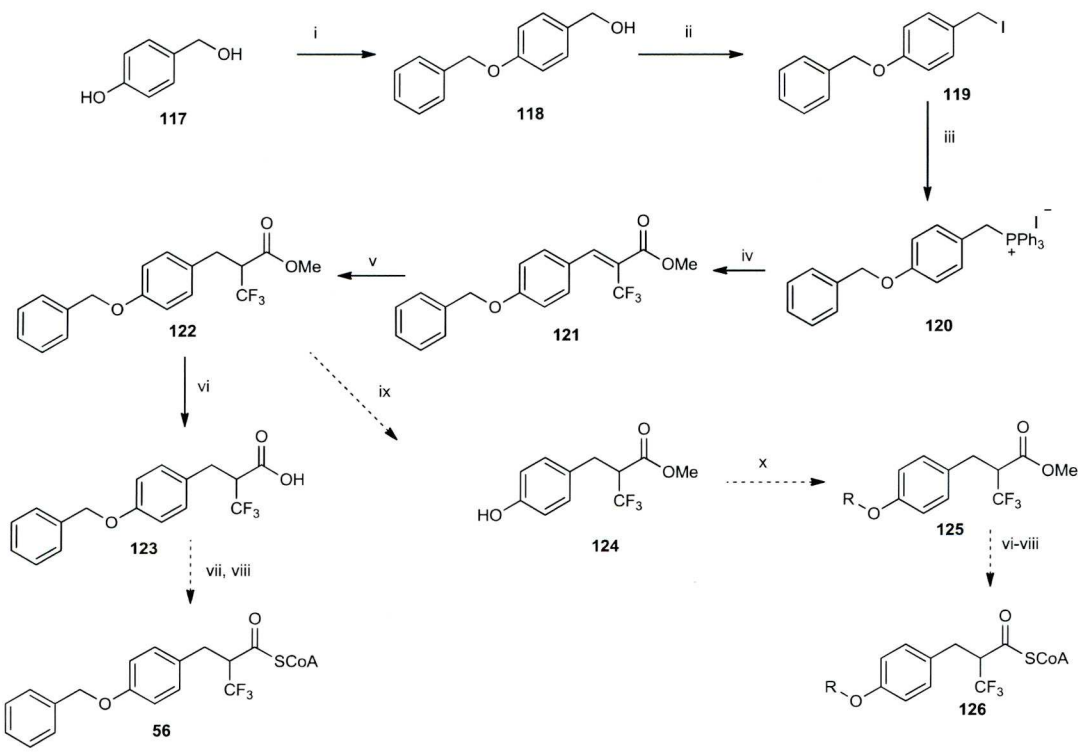


Fig 2.11 *Reagents and conditions*: i, K_2CO_3, 4-OHC_6H_4Br, acetone, 60°C, 4h, 91%; ii, PPh_3, Imid, I_2, DCM, 25°C, 30 mins 90%; iii, PPh_3, Tol, Δ, 3h 90%; iv, n-BuLi, $CF_3C(O)C(O)OCH_3$, THF, -78°C, 2h 45%; v, Pt-C, H_2, MeOH, 800 psi 95%; vi, LiI, DMF, 160°C, 8h, 63%; vii, CDI, THF, rt, 2h; viii, CoA-SH, $(Li^+)_3$ THF/H_2O, rt, 24h; ix, Pd-C, H_2, 5h, rt; x, K_2CO_3, Alkyl-X, acetone, 60°C.

2.6 Synthesis of β-trifluoroibuprofenoyl-CoA (60)

Work by Carnell *et al.*, has already ascertained that Ibuprofen (**22**) (despite being a known substrate of AMACR) has a comparible *Ki* value to that of the mono-fluorinated analogues of branched chain fatty acids when assayed against THC-CoA (**9**). This fact, alongside the fact that fluorinated analogues of the aliphatic substrates exhibit a 150 fold decrease in K_i compared to their non fluorinated analogues (based on two assays) lead us to envisage that fluorinated versions of Ibuprofen may exert excellent inhibition characteristics. This is further supported by the fact that (*R*)-ibuprofenoyl-CoA (**22b**) has a better affinity to AMACR than all known natural substrates (Table 2.1).

Substrate	Kd (MCR) (μM)	Kd (AMACR) (μM)
(*R, S*)-Ibuprofenoyl-CoA	24.5	56
(*S*)-Ibuprofenoyl-CoA	35.7	
(*R, S*)-THCA-CoA	26	60
(*R, S*)-Pristanoyl-CoA	41	76
Acetoacetyl-CoA	715	
Acetyl-CoA	585	

Table 2.1 Relative binding affinities of a range of natural substrates of MCR and rat AMACR.[61]

β-Trifluoroibuprofen (**134**) had been previously synthesised some 17 years ago by a rather convoluted method using chloropentafluoroacetone (**128**) as the starting material (Fig 2.12).[142] This CFC is now prohibitively expensive. A Friedel Crafts reaction was performed with the chloropentafluoroacetone (**128**) and isobutylbenzene (**127**). This was followed by chlorination and reduction to yield an alkene 1-isobutyl-4-(perfluoroprop-1-en-2-yl)benzene (**132**) which was then attacked by sodium ethoxide in ethanol to yield the penultimate intermediate (**133**) (in a 1:1 mixture of stereoisomers) and a side product. The product/side product ratio was not discussed. The saturated intermediate (**133**) was then converted to the desired product, β-trifluoroibuprofen (**134**). A more recent method employed the use of electrochemical carboxylation of a chlorinated species.[143] Both these methods are impractical in terms of large scale synthesis.

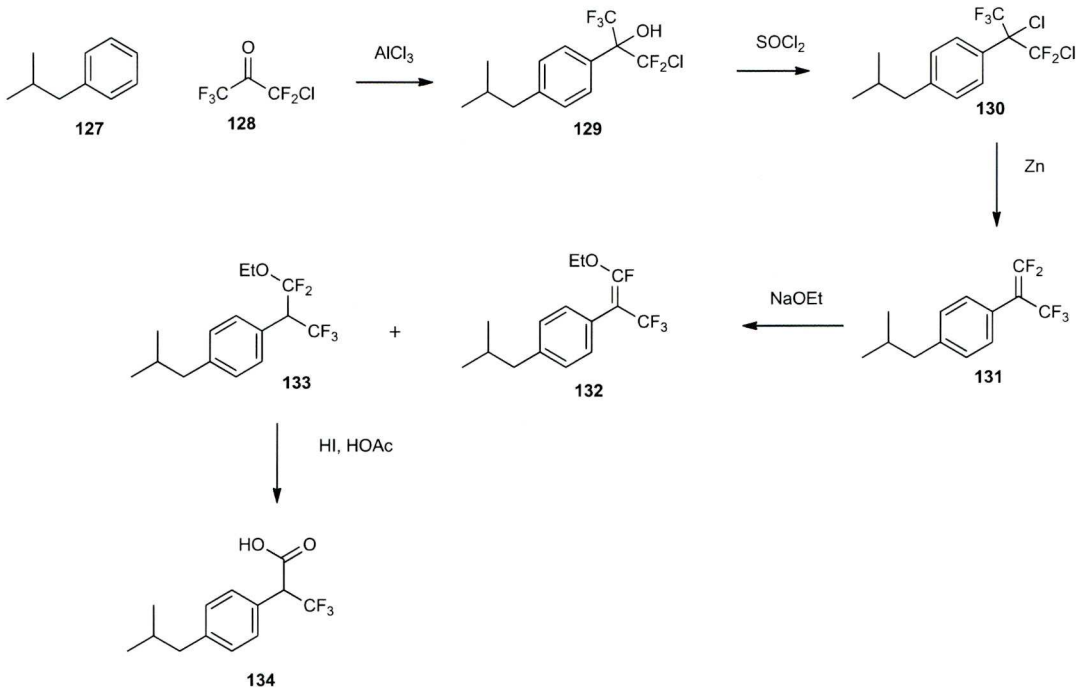


Fig 2.12. Previous synthesis of β-trifluoroIbuprofen by Middleton *et al.*

Our initial approach for the formation of β-trifluoroibuprofenoyl-CoA (**60**) was to use an analogous route employed by Shieh *et al.,* for the formation of ^{13}C-Ibuprofen, for metabolism studies with rat liver mitochondria[144] (Fig 2.13).

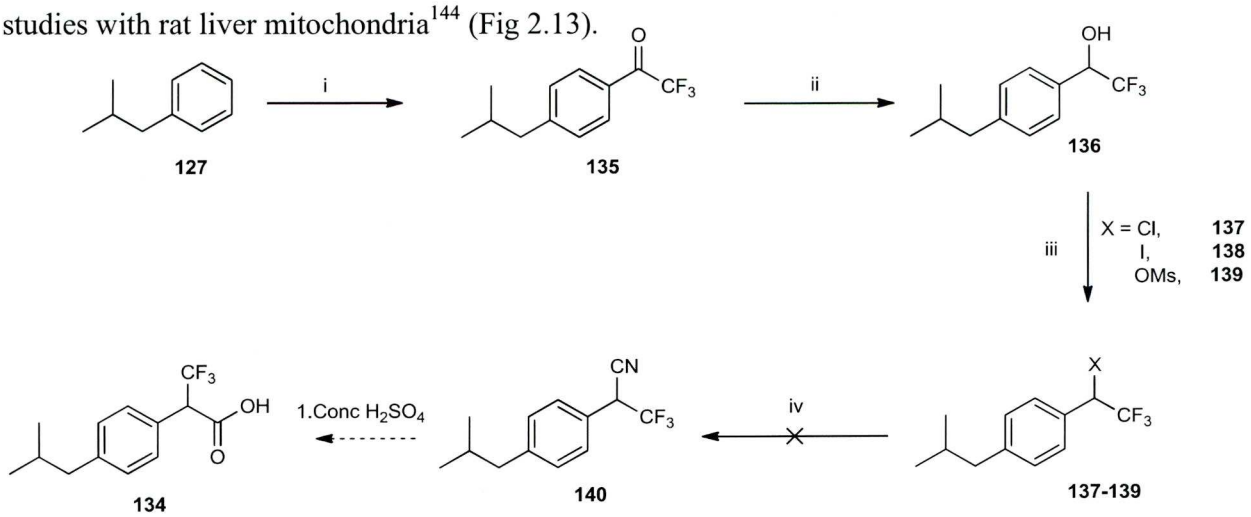


Fig 2.13. *Reagents and conditions*: i, $AlCl_3$, TFAA, DCM, 0°C, 1h, 74%; ii, $NaBH_4$, MeOH, 0°C, 1h; 90% iiia, $SOCl_2$, 80°C, 4h, 80%; iiib PPh_3, Imid, I_2, DCM, 40°C, 74%; iiic MsCl, NEt_3, DCM, 0°C, 88%.

First a Friedel Craft acylation reaction was performed with isobutylbenzene (**127**) and trifluoroacetic anhydride.[145] Although this is not particularly atom efficient, with the release of one equivalent of trifluoroacetic acid, the alternative, trifluoroacetyl chloride, is

impractical as its boiling point is -27°C. Iron chloride was initially used as it is a mild Lewis acid, but it was found that the reaction time was longer, yields were inferior and some *ortho* acylation product formed in comparison to the use of aluminium chloride. Upon formation of the acetophenone derivative (**135**), reduction was carried out by using sodium borohydride at 0°C. This reaction occurred almost instantaneously. The trifluoromethyl group leaves the ketone much more electronically exposed to nucleophilic attack. The next step in this sequence was to convert the hydroxy (**136**) into a better leaving group. The first group tested in this replacement was a chlorine (**137**) (as reported by Shieh *et al.*). In our hands, chlorination took longer and occurred at elevated temperatures. A chloro-cyano exchange was then attempted with use of sodium cyanide in DMSO at 80°C but failed. Repeating this reaction at higher temperatures still yielded no product although no decomposition had occurred and the starting material could be recovered. We then decided to try a range of better leaving groups. Mitsunobu chemistry was used to incorporate an iodine (**138**) which proceeded reasonably well, but again it was noted that the temperatures required to achieve conversion were elevated from that of normal hydroxyl-iodo displacement. Repetition of the cyanide incorporation again did not yield the desired product.

We then made the mesylate derivative (**139**) from the alcohol but cyanide displacement proved fruitless. Changing the cyanide counter ion from sodium to potassium did not yield the product, nor did changing the solvent to DCM or introducing 18-crown-6 to complex the potassium ion. Use of acetone cyanohydrin was also ineffective.

We can hypothesise why this cyanide insertion failed. The hydroxyl group can be displaced through an internal nucleophilic substitution mechanism. When using thionyl chloride,[146] this first reacts with the alcohol to form an alkyl chloro sulphite (**141**), which forms an intimate ion pair. The second step is the concerted loss of a sulfur dioxide molecule and partial internal replacement by the chloride (**137**), which was attached to the sulfite group in an S_Ni mechanism (Fig 2.14). The difference between S_N1 and S_Ni is that the ion pair is not completely dissociated, and therefore no real carbocation is formed,[146] which would otherwise be unstable because of the adjacent trifluoromethyl group. This rationale is also true in the formation of the iodo compound.

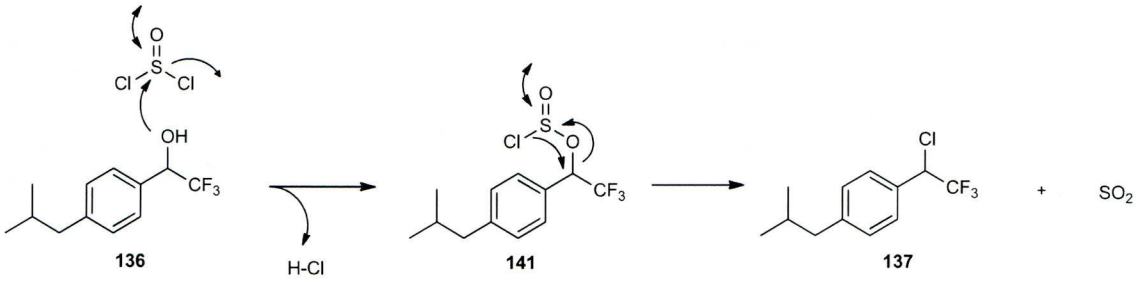


Fig 2.14 Mechanism of S_Ni chlorination.[146]

The direct S_N2 displacement of a mesylate group with cyanide group was not observed. This could be attributed to the steric effects or that the benzylic centre is deactivated by the CF_3 group such that only intramolecular delivery of a nucleophile is possible.

We then moved onto cyanohydrin chemistry. From the fluorinated acetophenone derivative (**135**) it is possible to produce a cyanohydrin (**142**) in two ways, either by acidifying an aqueous solution of sodium cyanide and the acetophenone to generate HCN *in situ*, or by the use of sodium bisulfite to activate the ketone,[147] followed by addition of sodium cyanide. The latter is more reproducible and safer. Upon purifying the cyanohydrins one should take much care as it can rapidly decompose back to the starting material (**135**) (Fig 2.15).

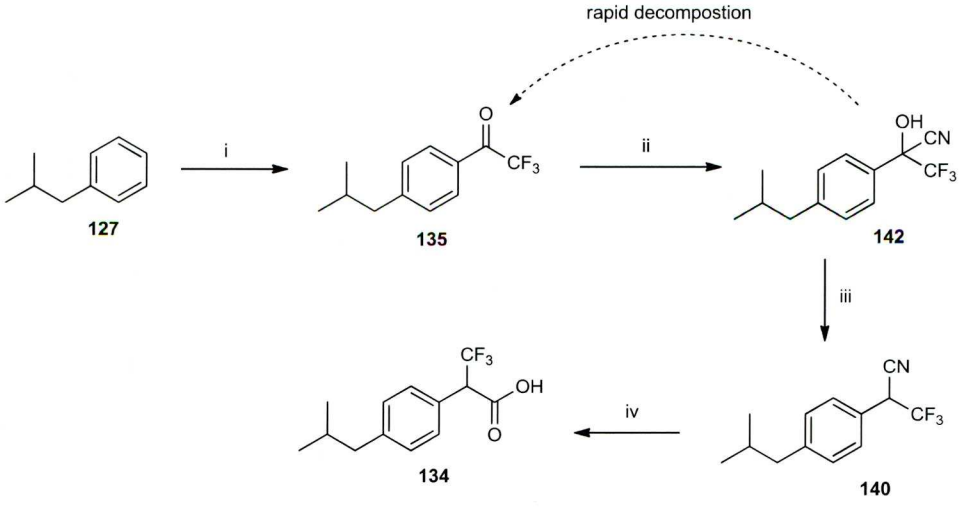


Fig 2.15 *Reagents and conditions*: i, $AlCl_3$, TFAA, DCM, 0°C, 1h, 74%; ii, NaCN, $NaHSO_3$, H_2O, rt, 1h, 30-50%; iii, P_4S_{10}, Tol, Δ, 4h, 58%; iv conc H_2SO_4, H_2O, 2h, 79%.

After formation and fractional distillation of the cyanohydrin, we then endeavoured to remove the hydroxyl group to give us our β-trifluoroibuprofen precursor (**140**). Stahly *et al.*

reported that refluxing phosphorus pentasulfide (**143**) in toluene with aromatic cyanohydrins yields the desired product[148] and indeed this was the case for our derivative. There is no literature mechanistic discussion on how this reagent brings about this transformation; in a recent review of phosphorus sulfide in organic chemistry this transformation is omitted altogether.[149] However, one can hypothesise that the mechanism may go through a thiol formation (**144**) from the phosphorus pentasulfide (**143**), followed by a disulfide formation (**145**), driven by the formation of the very stable P=O bond (Fig 2.16). To formally ascertain this, introduction of a deuterium atom on the hydroxyl group would indicate whether the same proton is reintroduced onto the product. Secondly, addition of an agent that suppresses the sulfur-sulfur bond formation such as DTT might stop the reaction at an intermediate stage. If there is evidence of a deuterium in a phosphorus (^{1}H decoupled) NMR, then we can be quite confident of this mechanism.

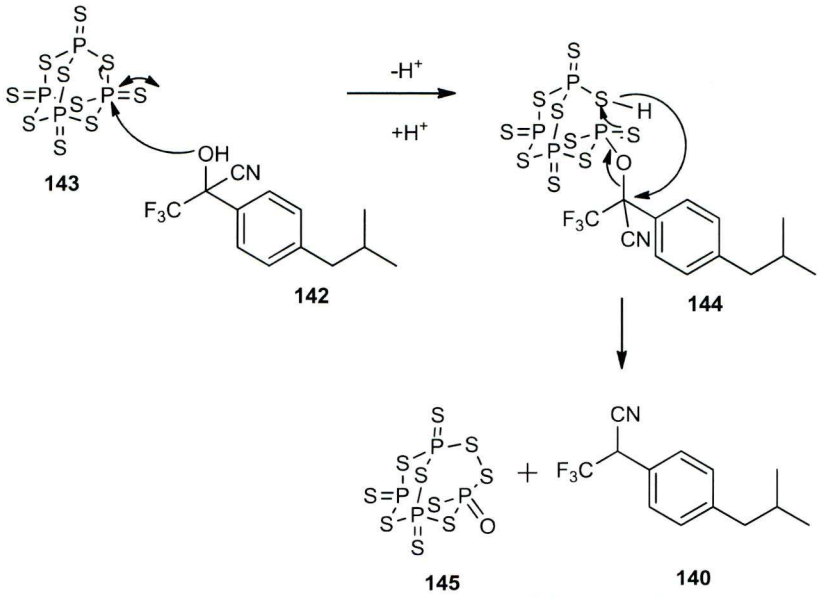


Fig 2.16. Proposed mechanism of phosphorus pentasulfide reduction.

After formation of the deoxygenated cyanide compound (**140**), we hydrolysed the nitrile with the use of a strong acid (sulphuric acid) to yield our desired compound (**134**). However, due to the capricious nature of the cyanohydrin formation, this was an impractical route and so another (more successful) route was devised (Fig 2.17). It should also be noted at this juncture that we were unable to obtain a high res mass spectrum of the free acid. However, we can suspect this to be the true product by performing ^{1}H NMR, ^{13}C NMR, IR and ^{19}F NMR. All of which are indicative of the desired structure.

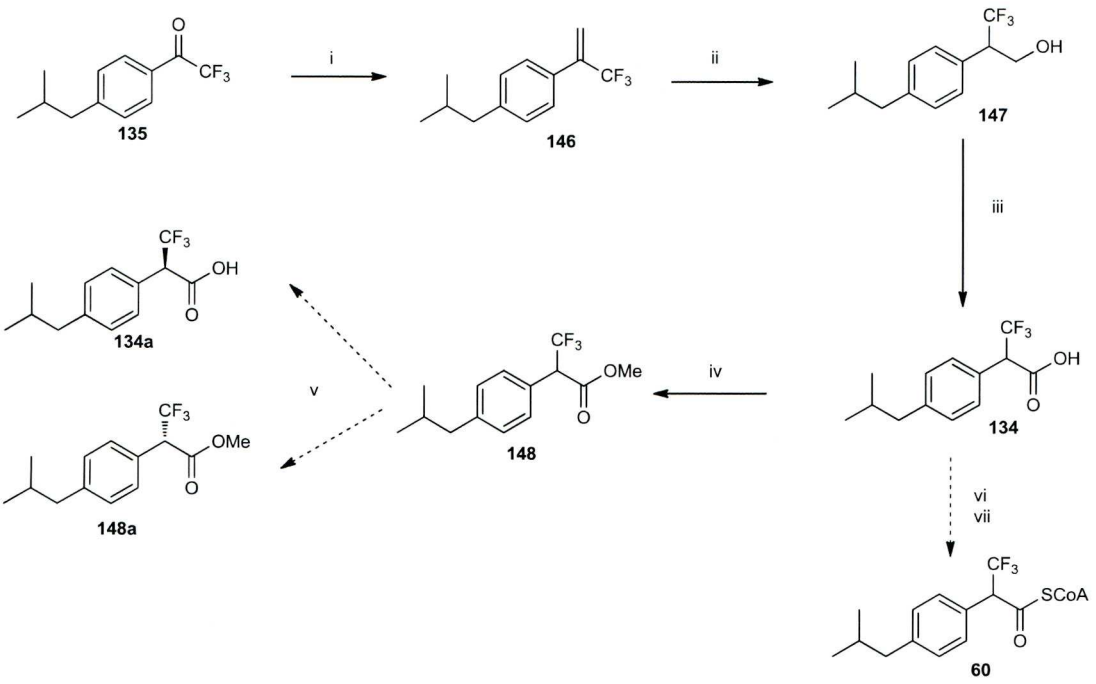


Fig 2.17. *Reagents and conditions*: i, *n*-BuLi, PPh_3MeI, -78°C, 39%; ii, $BH_3.SMe_2$, DME, 90°C, ON then H_2O_2, NaOH, 0°C 30 mins, 69%; iii, CrO_3, Me_2CO, rt, 2h; iv, MeOH, cat H_2SO_4, Δ, 99%; v, *Mucor Jarvinicus*, pH 7 buffer; vi CDI, THF, rt, 2h; vii, CoA-SH, $(Li^+)_3$ THF/H_2O, rt, 24h.

From the acetophenone derivative (**135**) (which was prepared as previously mentioned), we performed a Wittig reaction with commercially available triphenylphosphine methyl iodide. The product was then hydroborated/oxidised by refluxing with borane dimethyl sulphide followed by oxidation with 30% hydrogen peroxide solution.[150] Subsequently, the primary alcohol (**147**) was oxidised with Jones' reagent to give our desired acid[151] (**60**) which can then be CoA thioestified. An interesting point about the hydroboration/oxidation process should be made at this juncture. A significant byproduct was observed in 30% yield. This byproduct was characterised as being 2-(4-isobutylphenyl)propan-1-ol (**153**). This is unexpected and there have been no previous reports as to this type of product forming with these reagents. A proposed mechanism is as follows (Fig 2.18):

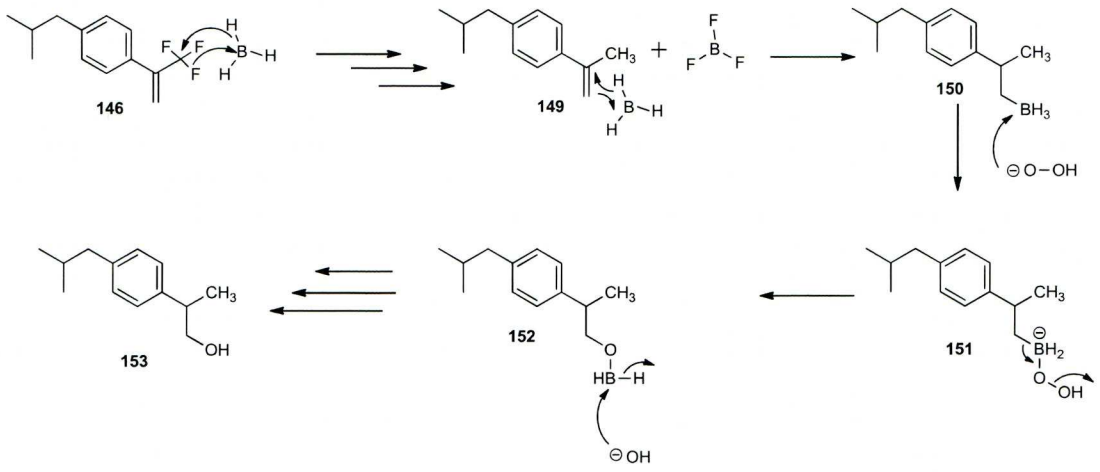


Fig 2.18 Proposed mechanism for the formation of 2-(4-isobutylphenyl)propan-1-ol.

It is possible that a competing reaction is occurring between the conventional hydroboration reaction and a hydrogen-fluoro exchange (**146/149**). It is anticipated that the hydroboration of the double bond is faster than the fluoro/hydrogen exchange of the trifluoro group. This explains why the dominant product is our desired compound. The driving force behind the side reaction is potentially due to the formation of the stronger B-F in comparison to the B-H (bond dissociation energies are 690 kJ/mol[152] and 440 kJ/mol[153] respectively), probably due to the orbital symmetry between the boron atom and the three similarly oriented p orbitals on fluorine atoms. Further experiments should be conducted to ascertain the merits of this proposed mechanism and to probe its synthetic value.

As mentioned at the beginning of the Section, the (*R*)-enantiomer of ibuprofenoyl-CoA (**22b**) has a lower K_i and better binding affinity then its (*S*) counterpart (**22a**). This inspired us to attempt the synthesis of diasteriomerically pure analogues of β-trifluoroibuprofenoyl-CoA (**60**). This was achieved by first esterifying the racemic free acid with the use of catalytic H_2SO_4 in MeOH and refluxing for 4 hours. A range of enzymes were then screened for enantioselective hydrolysis (Table 2.2).

Enzyme	Active	Enantioselective
Europa Lipase 15	No	--
Europa Lipase 1	Yes	No
Lipase F Ap15	No	--
Europa Lipase 20	No	--
Lipase AK	No	--
Lipase PS Amano	No	--
Novazyme KBS	No	--
Chirozyme L2	Yes	No
Chirozyme L3	No	--
Lipase 9 Amano 50	No	--
Mucor jarvinacus	Yes	Yes

Table 2.2 Enzyme screening for enantioselective hydrolysis of β-trifluoroibuprofenoyl-CoA.

Only three enzymes showed activity, *Europa Lipase 1*, *Chirozyme L2* and *Mucor jarvinicus* with only the latter showing enantioselectivity. Unfortunately the data for this eantioselectivity is missing. However Kato *et al.*, achieved stereoselective de-esterification of α-(Trifluoromethyl)-1H-indole-3-acetic acid ethyl ester,[154] which was achieved with 35% yield after 20 hours and 98% *e.e* of the free acid ($E > 100$). Although these two substrates are quite unrelated, there are similarities in the ester region.

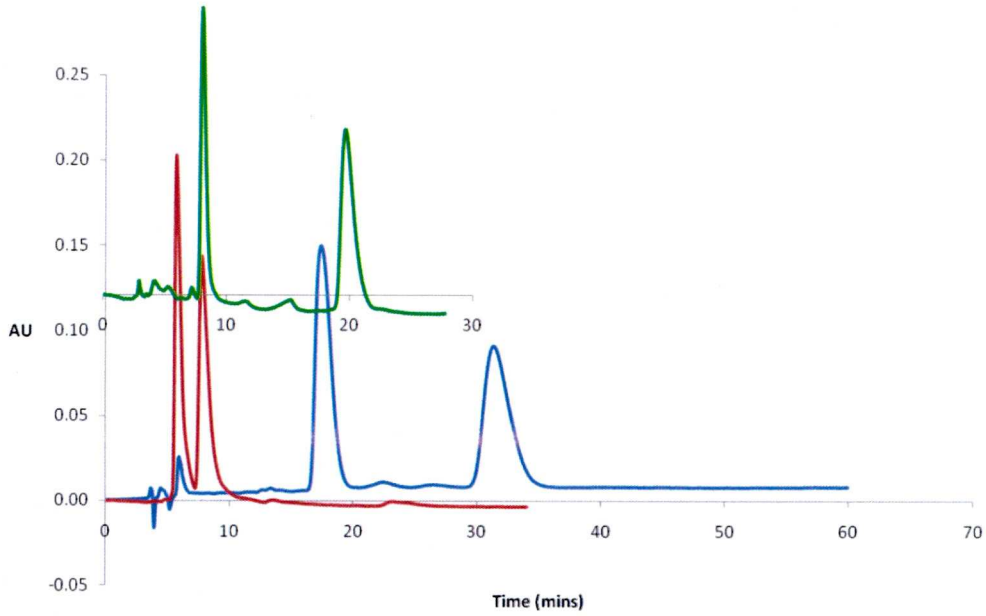


Fig. 2.19 HPLC traces of β-trifluoroibuprofen (blue) and β-trifluoroibuprofen methyl ester (red). Peaks at 5.6 and 7.7 mins correspond to the methyl ester, peaks at 17.2 and 31.6 mins correspond to the free acid. By comparing the relative retention times of the C*andida rugosa* promoted enantioselective hydrolysis of ibuprofen methyl ester (green), the (*R*)-enantiomer in each case has the later retention time. For column conditions – see experimental.

2.7 Chlorinated Ibuprofenoyl-CoA derivative (64)

The rationale behind the synthesis of α-chloroibufenacoyl-CoA (**64**) is similar to that as the α-chlorinated aliphatic chain (Section 2.4), being that the chlorine atom acts as an isosteric replacement of the natural α-methyl group, and that its electronegativity lowers the α-protons p*K*a. The further advantage of using the Ibuprofen derivative, as opposed to the aliphatic derivate, is that the side chain appears to have a higher binding affinity for the enzyme (*vide supra*), thus possibly increasing its inhibition potential. The synthesis began with a Friedel Crafts reaction of isobutylbenzene with ethyl 2-chloro-2-oxoacetate to give ethyl 2-(4-isobutylphenyl)-2-oxoacetate (**154**) which was subsequently reduced to the α-hydroxy ester **155**. Ten equivalents of thionyl chloride in DCM were used to chlorinate the species which was hydrolysed using a combination of hydrochloric acid and sulphuric acid[155] (base mediated hydrolysis could result in the S_N2 removal of the chloro group, yielding 2-hydroxy-2-(4-isobutylphenyl)acetic acid *via* chloride displacement. This free acid (**157**) was then activated with CDI, followed by coupling with coenzyme A lithium salt (Fig 2.20).

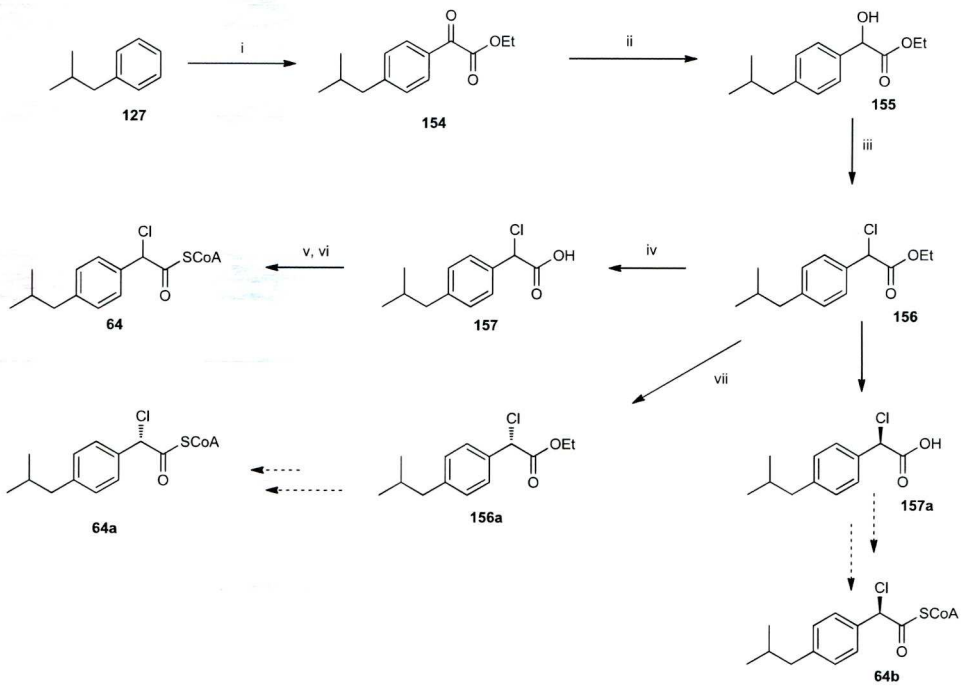


Fig 2.20. *Reagents and conditions*: i, $AlCl_3$, ClC(O)C(O)OEt, DCM, 0°C, 1h, 71%; ii, $NaBH_4$, MeOH, 0°C, 1h, 89%; iii, $SOCl_2$ 80°C, 2h, 90%; iv, HCl, H_2SO_4, H_2O, Δ, 2h, 73%; v, CDI, THF, rt, 2h; vi, CoA-SH, $(Li^+)_3$ THF/H_2O rt, 24h; vii, pH 7 buffer, *Candida Rugosa*, 48h, rt, 90% ee for the (*R*) acid.

Enantioselective hydrolysis of the racemic ethyl ester (**156**) was also achieved with use of C*andida rugosa* (see appendix 6) This is equivalent to *Candida cylindracea* used by Haughton *et al.,* for enantioselective hydrolysis of 1-chloro-2-aryl acetic acid esters (35% conversion, 85% *e.e*).[156] This yielded (*S*)-α-chloro-ibufenac ethyl ester (**156a**) with 99% e.e which can be hydrolysed and thioesterified in the usual fashion (Fig 2.20).

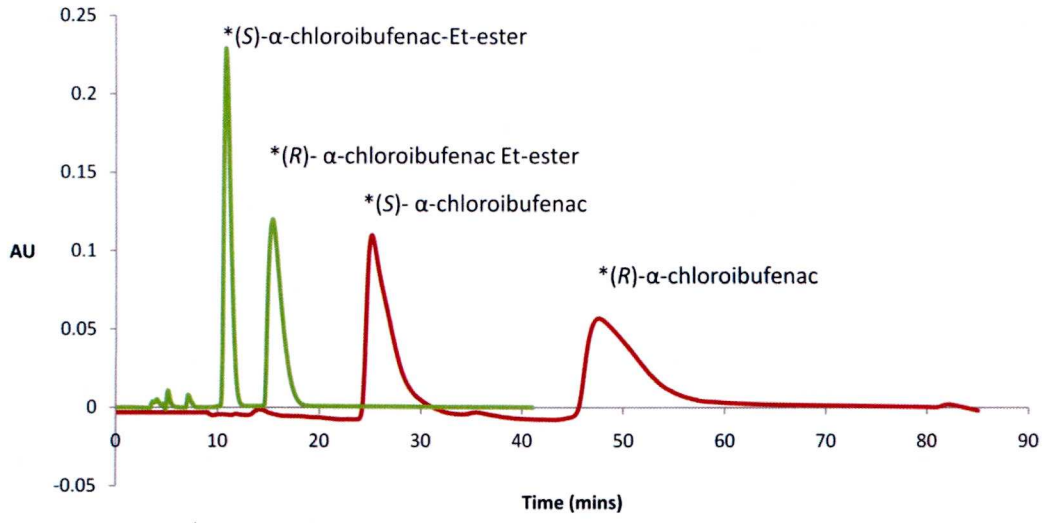
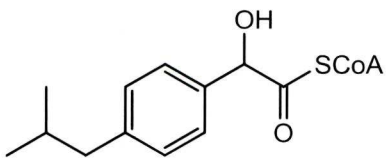


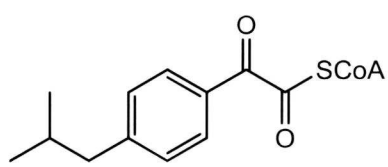
Fig. 2.21 HPLC traces of α–chloroibufenac (red) and α–chloroibufenac ethyl ester (green). Peaks at 10.9 and 15.4 mins correspond to the methyl ester, peaks at 25.2 and 47.7 mins correspond to the free acid. By comparing the relative retention times with ibuprofen and ibuprofen methyl ester, the (*R*)-enantiomer in each case has the later retention time. For column conditions – see experimental.

2.8 Further analogues of Ibuprofenoyl-CoA

From the route stated in Section 2.7, we have made a number of alternative analogous precursors to modified Ibuprofenoyl-CoA derivatives. Two are depicted below (Fig 2.22). The most obvious analogue is the conversion of the α-hydroxy ethyl ester (compound **155**, Fig 2.21) to the corresponding coenzyme A thioester (**158**). From the ethyl ester, we directly hydrolysed with 5*N* NaOH in MeOH/THF and thioesterfied in the usual fashion.



158



159

Fig 2.22 Other variations of Ibuprofenoyl-CoA.

The NMR chemical shifts of the α-protons of these various Ibuprofen derivatives all differ. This is likely to be relative to the acidity of the α-proton, arising from the de-shielding affect of the α-functional group. (Fig 2.23).

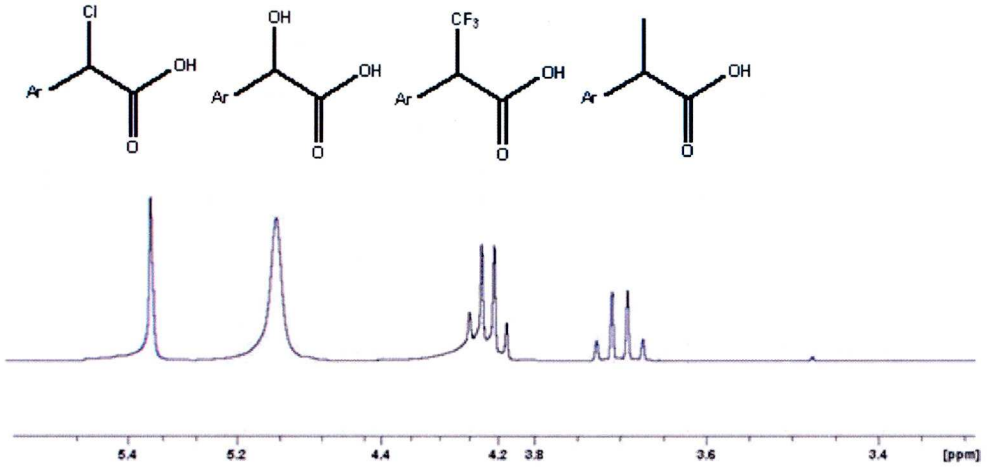


Fig 2.23 Relative positions of the α-proton in the NMR spectra of various Ibuprofen analogues.

Despite fluorine being the most electronegative element, it is obvious from Fig 2.23 that the CF_3 group does not exert the largest effect on the α-proton. It is clear that the greatest downfield shift is seen for the α-chloro compound. If the apparent inhibitory effect of the α-trifluorotetradecanoyl-CoA is due to the increased acidity of the α-proton, then it is reasonable to assume that the α-chloro derivative should give rise to improved inhibition.

We also synthesised 2-(4-isobutylphenyl)-2-oxoacetic acid (**159**) (Fig 2.22). Since the quaternary structures of formyl-CoA transferase and AMACR are very similar, and both involve the use of Asp as a catalytic residue, then the Asp function of AMACR could mimic the function of Asp in FRC. In the catalytic mechanism of FRC, Asp169 acts as a nucleophile, forming a mixed anhydride with formyl-CoA. It is therefore possible that Asp152 in AMACR, could act as a nucleophile on the α-ketone (Fig 2.24). If it exhibits good inhibition characteristics, then we could postulate such a mechanism.

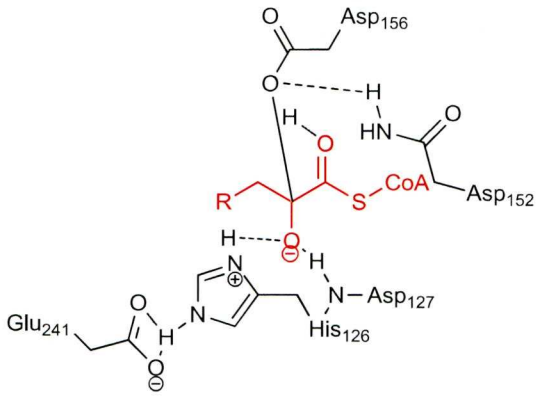


Fig 2.24 A pictorial representation of the possible mode of binding of 2-(4-isobutylphenyl)-2-oxoacetoyl-CoA.

2.9 Using extended conjugation as an approach for inhibition

The next potential inhibitor to be synthesised was (*E*)-2-methyl-4-(4-nitrophenyl)but-3-enoyl-CoA (**63**) (Fig 2.25). We anticipated that an enolate intermediate formed at the active site would be stabilised by extended conjugation, thus promoting inhibition.

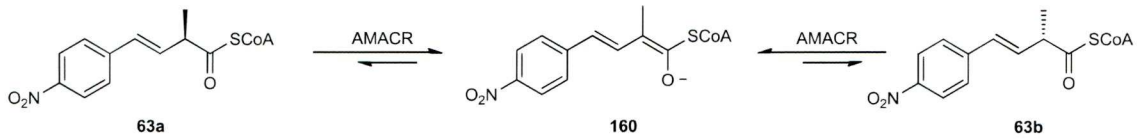


Fig 2.25 Enzyme catalysed formation of a stable, conjugated enolate from the potential inhibitor 63a/b.

The originally devised route to this compound was *via* a Wittig reaction (Fig 2.26). 3-Bromopropionic acid ethyl ester (**161**) was converted to a phosphonium salt (**162**) by refluxing with triphenylphosphine in toluene. The Wittig reaction was then attempted using *n*-BuLi as a base and 4-nitrobenzaldehyde.

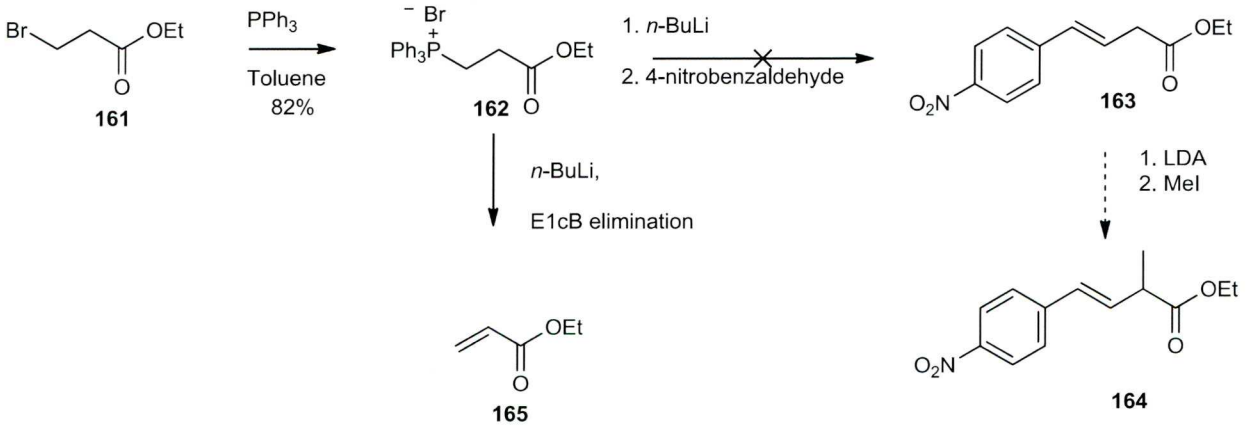


Fig 2.26 Initial route for (*E*)-2-methyl-4-(4-nitrophenyl)but-3-enoyl-CoA synthesis.

The required product was not formed. It was noted however that triphenylphosphine was released. This can be rationalised by the irreversible elimination of the PPh_3. The p*K*a difference between the α-and β hydrogens (relative to the phosphorus) is relatively small, with a difference of around 5. Although the proton adjacent to the phosphorus atom is more acidic, the irreversible nature of the elimination of the PPh_3 renders this E1cB elimination more likely.

An alternative was to perform an Aldol reaction with acetone and 4-nitrobenzaldehyde in the presence of sodium hydroxide (Fig 2.27). The product was then dehydrated by refluxing in acidic aqueous medium. This α,β-unsaturated ketone (**167**) was then reduced to the corresponding alcohol (**168**) using Luche conditions and converted to the chlorinated analogue (**169**) (with use of neat thionyl chloride). Chloride displacement was then attempted with use of NaCN in DMSO at 90°C but unfortunately proved fruitless.

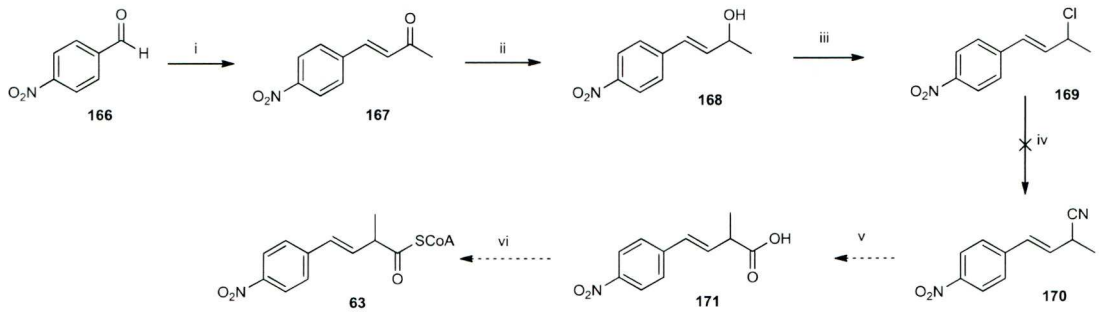


Fig 2.27. *Reagents and conditions*: i, NaOH, Me_2CO, rt, 1h then H_2SO_4, H_2O, 100°C, 2h; 93%; ii, $CeCl_2$, $NaBH_4$, 0°C, MeOH, 1 h, 86%; iii, $SOCl_2$, 80°C, 2h 90%; iv, NaCN, DMSO, 90°C, ON, no reaction.

From analysing the reaction performed, it was noted that little of the starting material had reacted. Although allylic halides are generally considered more reactive then their non allylic counterparts, the *para* nitro aromatic group possibly destabilises build up of positive charge required for displacement of the chlorine atom. Furthermore, from the NMR of the crude reaction mixture, the aromatic region had changed from a doublet of doublets to a complex mixture. This is possibly due to the von Richter reaction which converts aromatic nitro compounds to aromatic carboxylic acids with the use of a cyanide ion. According to the literature, the von Richter reaction works best when there are electronegative substituents on the ortho and/or para positions. Yields are always poor (<50%) and a notable amount of 'tar' is formed.[157]

We then decided to perform a Willgerodt Kindler reaction on compound **167** with lead acetate and borane trifluoride dietherate to give us the unsaturated ester (**172**).[158] We envisaged that the α-methyl group could be incorporated by the use of LDA and methyl iodide. We could then hydrolyse the methyl ester to the corresponding acid and then thioesterify as before (Fig 2.28). At present, the synthesis of this compound is at the point of α-methylation. However, we do not envisage a problem with the subsequent steps. Of course there is the possibility of γ-methylation, but this can be controlled. Typically α-electrophilic addition is kinetically favoured,[159] and is the preferred carbon of attack for soft electrophiles[160] (with methyl iodide being a moderately soft electrophile).

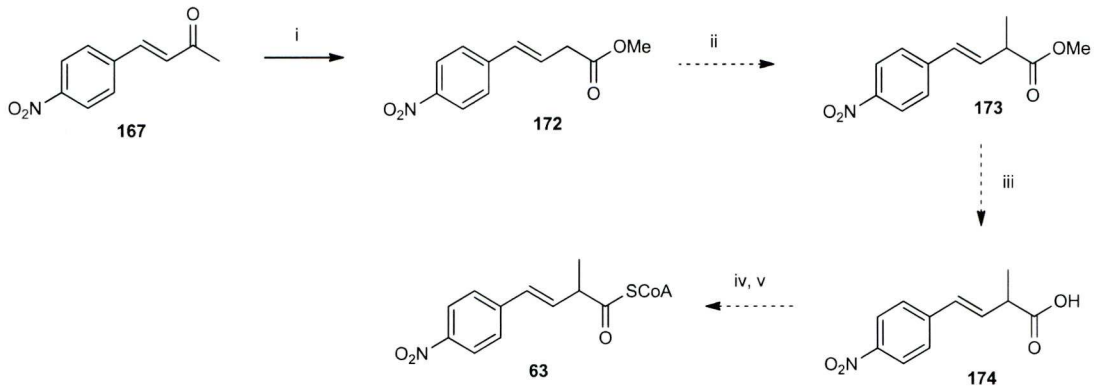


Fig 2.28. *Reagents and conditions*: i, $Pb(OAc)_4$, $BF_3.OEt_2$, MeOH, Tol, rt, 24h, 55%; ii LDA, DMPU, -78°C, then MeI; iii, 5N NaOH, MeOH/THF, iv CDI, THF; v, CoA-SH, $(Li^+)_3$, THF/H_2O, rt, 24h.

2.10 α-fluorinated substrate as a mechanistic probe (62).

The synthesis of α-fluoroibuprofenoyl-CoA (**62**) will enable us to conclusively ascertain whether the reason for the inhibition by α–trifluoromethyltetradecanoyl-CoA (**49**) is because of the lowering of the pKa of the α–proton or because of an increased affinity of the substrate for the active site. The α-fluoro atom (**62**) is unlikely to be removed when (if) bound to AMACR. Therefore we can directly compare the K_i value for α-fluoroibuprofen with Ibuprofen for inhibiting the racemisation of 2-methyl myristoyl-CoA (an analogue of the natural substrate) by AMACR. If Ibuprofenoyl-CoA (**22**) has a lower K_i value than α-fluoroibuprofenoyl-CoA (**62**), then this would suggest that the mechanism of inhibition by our known inhibitors is in fact due to the lowering of the p*K*a of the α-proton and the facilitation of the formation of the enolate. If however the K_i values are similar, then this

would indicate another mode of inhibition, probably due to the increased affinity of the substrate (inhibitor) for the active site of the enzyme. The synthesis of α-fluorinated ibuprofen (**62**) began with the esterification of racemic Ibuprofen to form Ibuprofen methyl ester (**176**). This then underwent α-fluorination with the use of LDA and NFSI.[161] The methyl ester was then hydrolysed[162] and thioesterified with coenzyme A lithium salt (Fig 2.29).

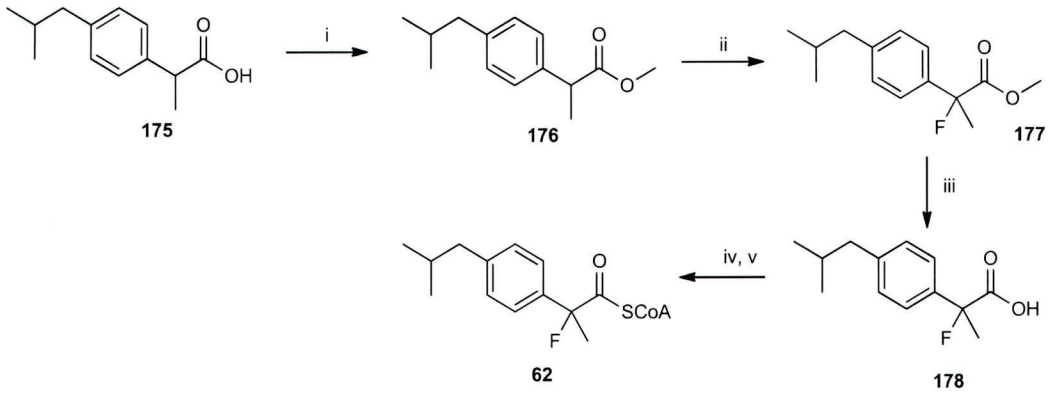


Fig 2.29 *Reagents and conditions*: i, MeOH, cat H_2SO_4, Δ, 4h, 99%; ii, n-BuLi, DIPEA, -78°C then NFSI, warmed to rt, 1hr 66%; iii, 5*N* NaOH, MeOH/THF, rt, ON 96%; iv CDI, THF, v, CoA-SH, $(Li^+)_3$, THF/H_2O, rt, 24h.

NFSI is generally considered a good source of F+, however, surprisingly it has never been used as a source of F+ for fluorinating 2-aryl propionic acid esters. Perhaps it was assumed too bulky to effectively fluorinate. Nonetheless, due to its stability and ease of use (due to it being in a crystalline state), it was tested, and pleasingly proved successful. The initial attempt at fluorination gave a yield of 75% (by NMR spectroscopy) with 15% unreacted starting material. Due to the similar nature of the product and starting material on the TLC plate, it was impossible to separate the mixture. We overcame this problem however by re-introducing the mixture to the same reaction conditions. This time there was no starting material seen by NMR.

2.11 Probing the necessity of an α–methyl group (**182**).

The rationale behind the synthesis of our final inhibitor/probe, 2-(4-isobutylphenyl)acetyl-CoA (**182**) was to probe the binding of Ibuprofen with and without the methyl group and thus demonstrate how much influence the α-methyl/aromatic region has on the inhibition of the enzyme. There is conflicting evidence on the extent of enzymatic turnover of straight chain aliphatic substrates.[82, 85] It would therefore be interesting to investigate whether the Ibuprofen derivative is bound to the active site. This can easily be ascertained using isothermal titration calorimetry. It could also give a better insight to the significance and influence of the interactions between the binding pocket and the 'R' group of the CoA thioester. The synthesis of the title compound was as follows:

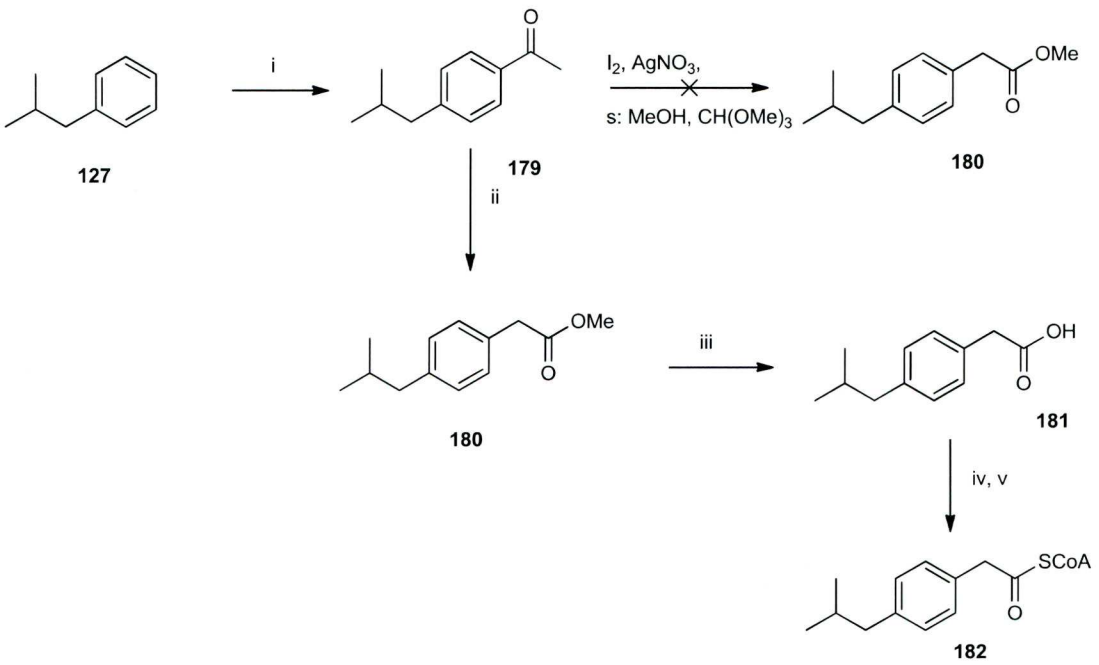


Fig 2.30 *Reagents and conditions*: i, $AlCl_3$ $(CH_3CO)_2O$, DCM, 0°C, 1h, 74%; ii, H_2SO_4, $PhI(OAc)_2$, MeOH, 70°C, 2h, 63%; iii, 5*N* NaOH, MeOH, rt, 3h, 94%; iv, CDI, THF, rt, 2h; v, CoA-SH, $(Li^+)_3$, THF/H_2O, rt, 24h.

Initially, a Friedel-Crafts reaction was performed with isobutyl benzene (**127**) and acetic anhydride to form the *para iso*butyl acetophenone (**179**). The initial Wilgerodt Kindler reaction with silver nitrate and trimethoxymethane in methanol did not yield the desired intermediate (**180**). However, performing a carbonyl migration and esterification with the use of diacetoxyiodobenzene proved successful.

Conclusion

To summarise, a range of aliphatic/aromatic inhibitors/precursors (Fig 2.31/2.32) have been synthesised using a range of different chemical techniques. Although we have a range of thioesters for testing, we have kept a larger number of potential inhibitors as their free acid/methyl ester precursors. Coenzyme A thioesters are notoriously difficult to store. We have found that after a short time in storage (at -20°C), they readily undergo hydrolytic cleavage. The hydrolysis of a thioester is thermodynamically more favourable than that of an oxygen ester. The C-S thioester bond is not stabilised in the same manner as a C-O ester bond due to the less efficient C-S pi interaction, rendering the thioester bond more reactive. This reactivity is increased by the fact that the neighbouring groups are often electronegative, further exposing the carbonyl carbon to nucleophilic attack. Moreover, the CoA moiety contains several other positions where cleavage can occur.

Figure 2.31 shows the available CoA esters synthesised as potential inhibitors. Figure 2.32 shows the available CoA ester precursors:

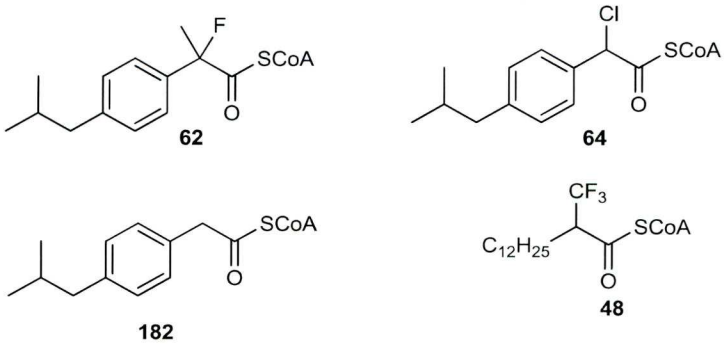


Fig 2.31 A range of coenzyme a thioesters stored in the freezer.

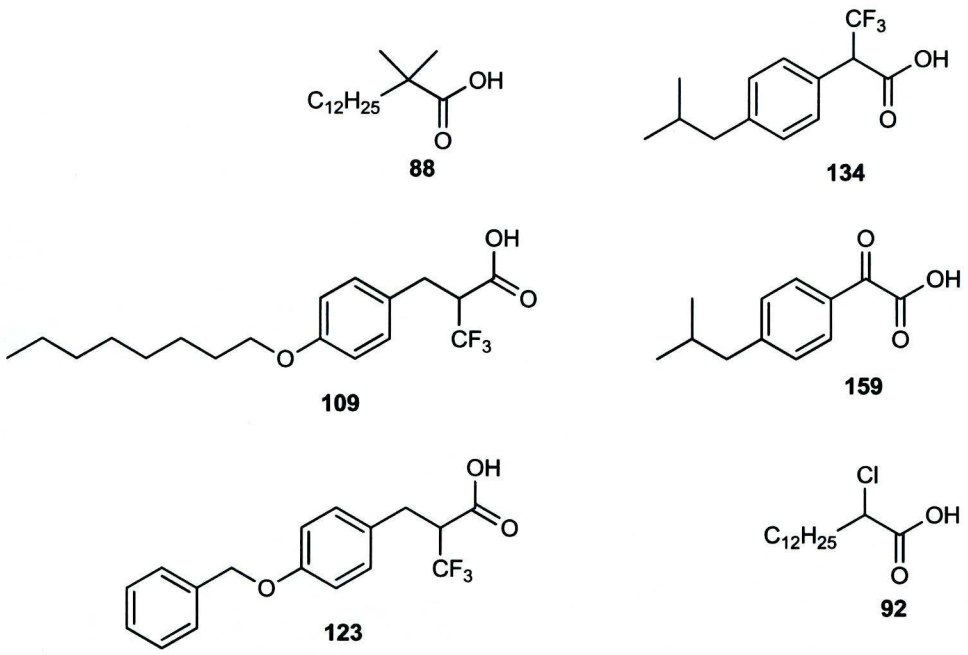


Fig 2.32 A range of potential inhibitor precursors.

In the next chapter we will discuss the synthesis of substrates for assaying purposes in order to test these inhibitors.

CHAPTER 3

SYNTHESIS OF SUBSTRATES

3.1 Project aims

As we now had a range of potential inhibitors to test against AMACR, it was important to obtain a reliable, convenient assay in order to test their efficacy. A range of different assaying systems and model substrates were explored and their synthesis is discussed herein.

3.2 Synthesis of HPLC assay substrates

As Ibuprofenoyl-CoA (**22**) is a known substrate of AMACR, we envisaged that an assay could be achieved by monitoring the conversion of enantiomerically pure (*S*)-Ibuprofenoyl-CoA (**22a**) to its racemate. The synthesis of the substrate began with the activation of (*S*)-Ibuprofen as its acyl imidazoyl derivative (analogous to Fig 2.02) which can be isolated and characterised. This was then thioesterified by the addition of coenzyme A lithium salt in a mixture of THF and water. The thioester was then purified by reverse phase HPLC (Zorbax C18 column, 250mm by 4.5mm) with an eluent system of 50mM potassium phosphate buffer (pH 6) and acetonitrile. However, upon lyophilization, a notable amount of salt precipitated. Therefore further purification through a desalting column was needed. Using ammonium acetate as a volatile buffer, a range of pH's were evaluated (see Appendix 1) and the optimum pH of the ammonium acetate buffer was pH 6. Above pH 7, significant hydrolysis was observed while at lower pHs, the amount of product was reduced. After purification, the ester was re-suspended in the ammonium acetate buffer in order to determine the quantity of material. This was achieved by measuring the UV absorption in 1ml of a buffered solution. The quantity of material was then calculated based upon the extinction coefficient of the adenosine moiety of the CoA substituent (λmax = 260nm, ε=15800 M^{-1} cm^{-1}). Thus it was possible to accurately calculate the quantity of product generated.[121]

To initially ascertain the enantiomeric purity, a small quantity of (*S*)-Ibuprofenoyl-CoA (**22a**) was subjected to base hydrolysis with sodium hydroxide. The resulting free acid was injected onto a chiral OJ column (250mm by 4.6mm) and the e.e. was obtained by measuring the relative areas of each peak of (*R*) and (*S*)-Ibuprofen (Fig 3.01). Following thioesterification and hydrolysis, no racemisation was observed. As this substrate became a focal point of the research, a ^1H NMR of the sample was obtained (see Appendix 2).

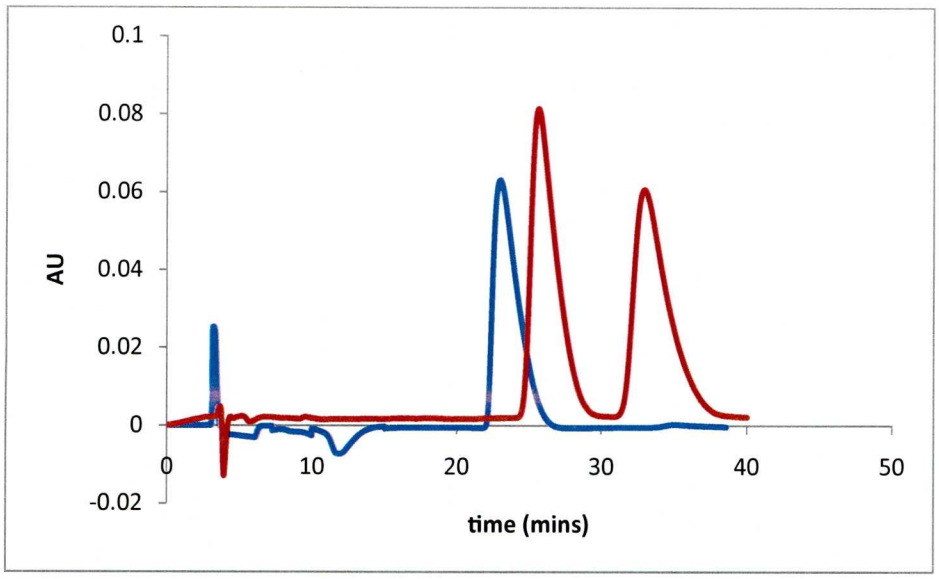


Fig. 3.01 HPLC trace of racemic Ibuprofen (red) and (*S*)-Ibuprofen (blue). Peaks at 22.76 (blue) and 24.6 (red) correspond to the (*S*)-enantiomer and the peak at 32.9 mins corresponds to the (*R*)-enantiomer respectively. Column: Chiral OJ (250mm by 4.6mm). For column conditions – see experimental.

3.3 Synthesis of LCMS assay substrates

An alternative LCMS based assay was also devised. This would enable us to monitor the conversion of α-deuterated Ibuprofenoyl-CoA (**67**) to its protonated form (Fig 3.02). It was envisaged that this could be correlated with rate of racemisation. The substrate synthesis began by esterifying racemic Ibuprofen (**177**) in methanol with a catalytic amount of H_2SO_4 for 4 hours. This was then α-deuterated by adding sodium methoxide to a solution of Ibuprofen methyl ester (**178**) in CD_3OD. This allowed us to monitor the conversion by directly subjecting the crude reaction mixture to NMR analysis. Once complete, this was hydrolysed with sodium hydroxide and thioesterified in the usual fashion.

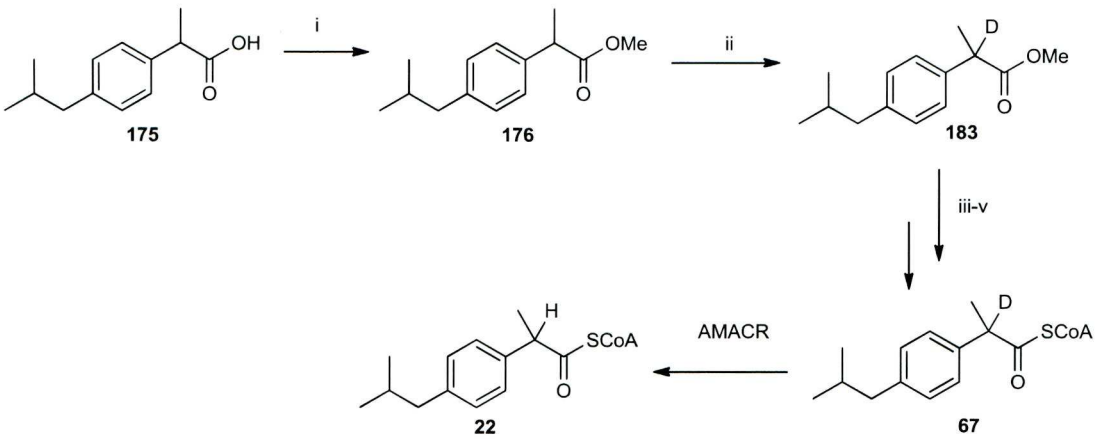


Fig 3.02 *Reagents and Conditions*; i, H_2SO_4, MeOH, 80°C, 4 h, 99%; ii, NaH, THF/D_2O, rt, 12h, 99%; iii, NaOH, MeOH, rt, 2h 99%; iv, CDI, THF, rt, 2h; v, CoA-SH, $(Li^+)_3$, THF/H_2O, rt, 24h.

Initially we used the same conditions for the LCMS assay as for the HPLC purification of Ibuprofenoyl-CoA (RP C-18 with an eluent system of 66:34 10mM ammonium acetate: MeCN respectively), which under the HPLC conditions gave a sharp peak at 5.3 mins. With the LCMS system however, the peak shape was broad and shouldered. Poor peak shape can be caused by the compound's reactivity towards the acidic silanols on the column - although this was suprising as the HPLC peak shape was sharp. Adding formic acid to the eluent (which is traditionally used to sharpen peak shape) suppressed MS ionisation altogether and no peak was observed.

Sidenius *et al.* tested the *in vitro* reactivity of Ibuprofenoyl-CoA with glutathione[163] using LCMS with an eluent system of 79:21 ammonium acetate: MeCN using a phenyl hexyl column. Initial testing of these conditions with our C-18 column gave the same peak shape. A phenyl-hexyl column has a stationary phase of terminal phenyls with hexyl (C6) linkers and is known to provide separations not achievable on C18 or C8 columns. Once a phenyl hexyl column had been obtained, Sidenius's conditions were used and the HPLC traces of (H)-Ibuprofenoyl-CoA (22) and α-deutero-Ibuprofenoyl-CoA (**67**) were sharp and unshouldered as can be seen from Fig 3.03.

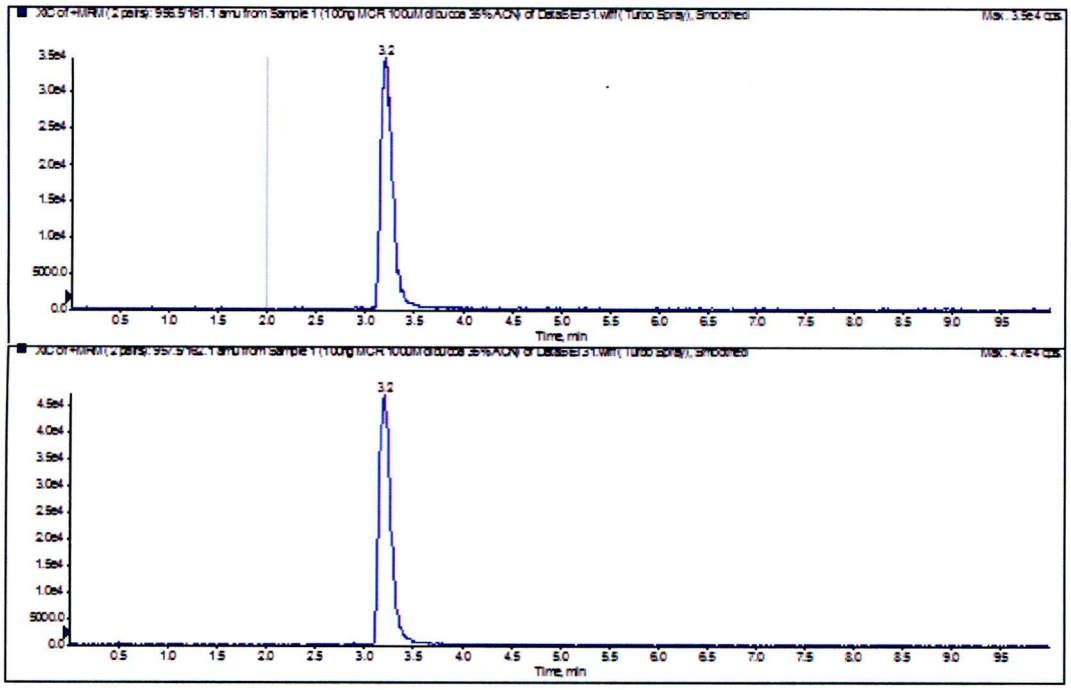


Fig 3.03 LCMS traces of (H)-Ibuprofenoyl-CoA (top) and α-deutero-Ibuprofenoyl-CoA (bottom). RT = 3.2 mins Column: Phenyl-hexyl (100mm by 4.6mm). Eluent system: 79% (v/v) 10mM ammonium acetate-acetic acid (pH 6): 21% (v/v) MeCN. 1ml/min.

3.4 Development of chromogenic assays

3.4.1 Synthesis of aromatic chromogen.

As discussed in Chapter one, we have endeavoured to develop a fast, reliable chromogenic assay which would enable us to screen thousands of potential inhibitors quickly and accurately. Our strategy was to design potential chromogens that incorporated the usual α–methyl group in addition to a group at the β–position that could leave under mild conditions catalysed by an active site base. The first group of chromogenic substrates included CoA thioesters which contain a *para*-nitro aromatic side chain. Upon formation of the chromophore (**71**), a UV max of 307nm would be observable (Fig 3.04).

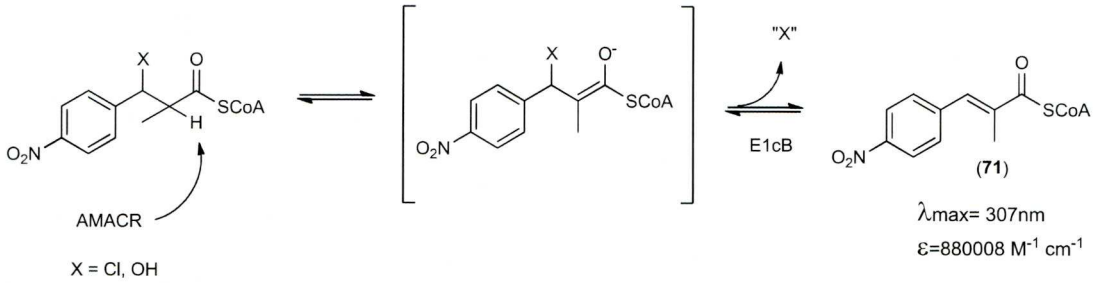


Fig 3.04. Proposed mechanism of enzymatically catalysed chromophore formation from chromogen.

Two different leaving groups were envisaged, chloro (**68**) and hydroxy (**67**). It was anticipated that the chloro derivative would be more prone to E1cB elimination than the corresponding hydroxy derivative. However, by using the X-ray crystal structure of ibuprofenoyl-CoA in MCR[61], it is reasonable to suggest that the β-hydroxy group can hydrogen bond with several enzymatic residues, which could aid in the E1cB elimination (Fig 3.05). Synthesis of the chromogenic substrates were designed to have no diasteriomeric preference. This will allow for quick analysis as to whether any diasterioisomer has activity.

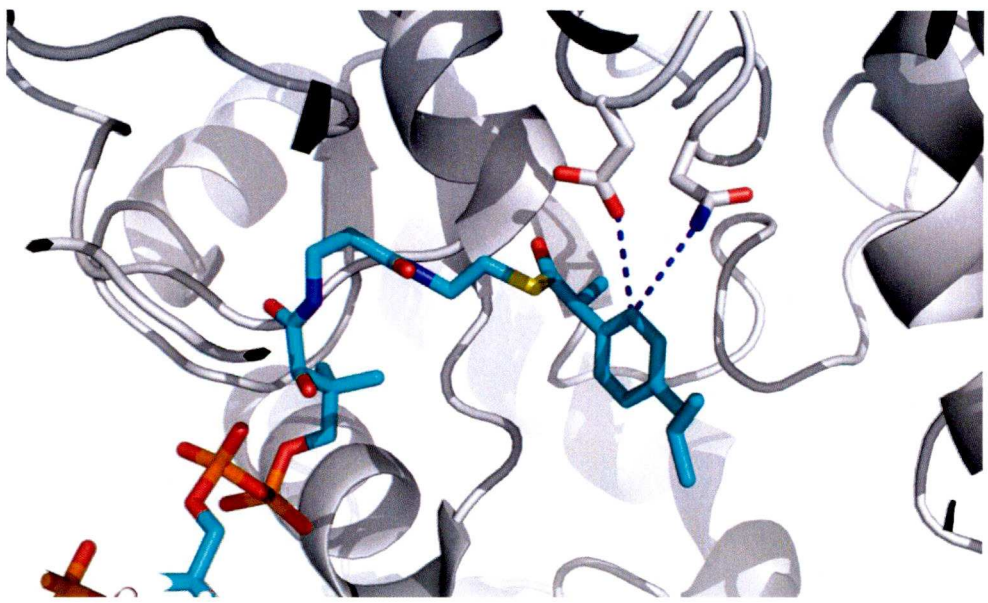


Fig 3.05. Pymol image of bound ibuprofenoyl-CoA to MCR. Adapted from *J. Mol .Biol.* **2007**, 367, 1145-1161. Hydrogen bonding between the proposed β-hydroxy thioester (**67**) as represented by the *ortho* aromatic carbon and residues Asp156 (left) and Asn152 (right) with bond lengths of 3.4 and 3.8Å respectively. Courtesy of Dr Rob Gibson, Biological Sciences, UoL.

The synthesis of the chromogenic substrates began with an aldol reaction between 4-nitrobenzaldehyde (**184**) and propanoic acid methyl ester (**185**) to give the α–methyl β-hydroxy ester (**186**). The hydroxy derivative was obtained by hydrolysing the ester with sodium hydroxide to give the free acid (**188**) which was then thioesterified. Alternatively, the preparation of β-chloro derivative was attempted by chlorination of the β-hydroxy ester with thionyl chloride and then de-esterification with hydrochloric acid (**189**) (as opposed to base hydrolysis that could possibly lead to elimination of the chloro group) (Fig 3.06).

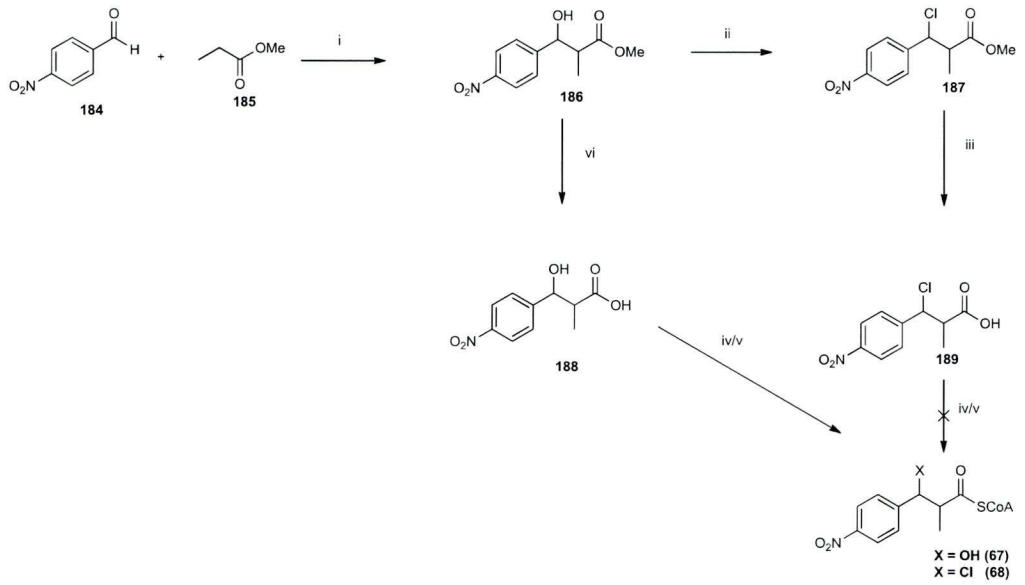


Fig 3.06 *Reagents and conditions*; i, LDA -78°C, THF, then 4-nitrobenzaldehyde, 70%; ii, $SOCl_2$, 80°C, DCM, 76%; iii, 5M HCl, H_2O, 100°C, 3h, 99%; iv, CDI, THF, rt, 2h; v, CoA-SH, $(Li^+)_3$ THF/H_2O, rt, 24h; vi, 1M NaOH, MeOH/H_2O, 0°C, 2h, 99%.

Thioesterification of the chloro derivative (**189**) was problematic. Although it was possible to obtain and isolate the acyl imidazolium intermediate (**190**); upon addition of coenzyme A, the chlorine eliminated to yield a highly conjugated thioester (**71**). A possible reason for the chlorine elimination may be due to the release of imidazole upon thioesterification. This may encourage removal of the α-proton resulting in E1cB elimination of the chlorine (Fig 3.07). This is unexpected as traditionally imidazole is a mild base, with a p*K*a of approximately 8 which conventionally would be too weak to remove this proton. However this proton may be sufficiently active in this case because of the electronegative nature of the chloro and thioester groups. The 3-hydroxy analogue (**67**) however does not suffer from the same elimination and was converted efficiently.

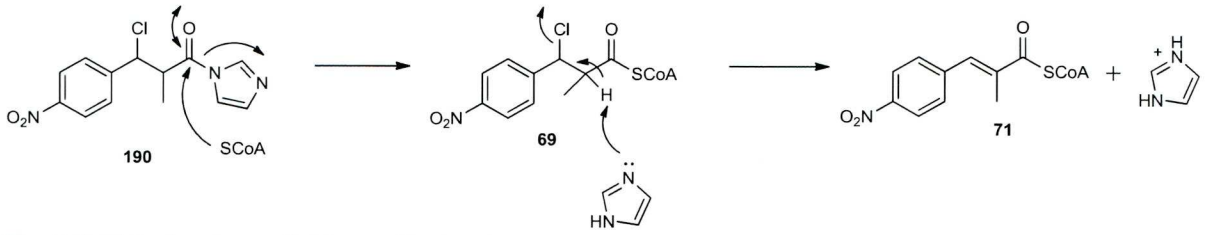


Fig 3.07 Mechanism of chloro elimination.

3.4.2 Synthesis of aromatic chromophore

The corresponding acyl-CoA was generated in order to have a standard sample for the UV chromophore we hoped to generate enzymatically. The synthesis began by Heck coupling 4-nitrobromobenzene (**191**) and methyl acrylate (**194**) (Fig 3.08) to produce (*E*)-methyl-2-methyl-3-(4-nitrophenyl)acrylate (**193**). The stereochemistry was ascertained by comparing the ^{1}H NMR of the aforementioned compound with (*E*)-ethyl-2-methyl-3-(4-nitrophenyl)acrylate (An ethyl ester derivative that has been previously characterised by ^{1}H NMR[164]). The yield of this reaction was very poor with an average yield of 22%.

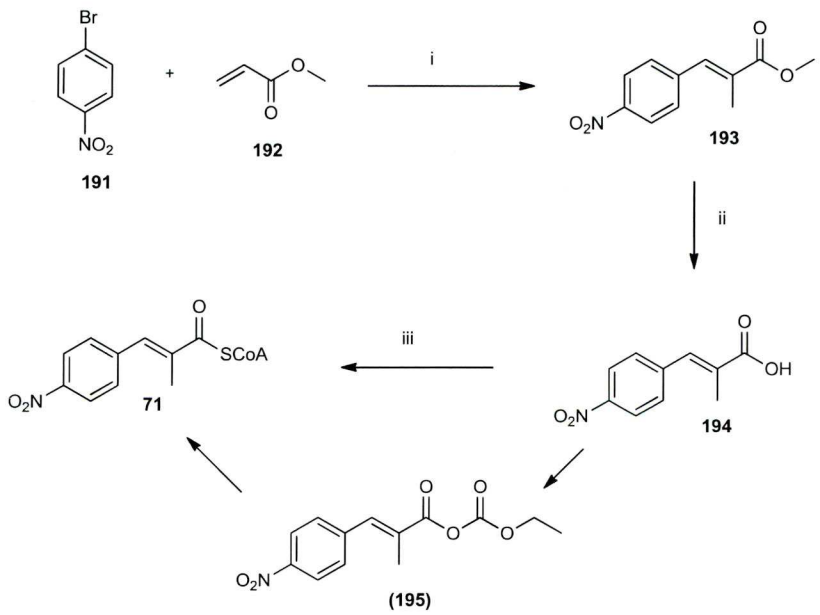


Fig 3.08 *Reagents and conditions*; i, $Pd(OAc)_2$, PPh_3, Na_2CO_3, 130°C, NMP 2h, 22%; ii, 5*N* NaOH, MeOH, rt, 72h, 83%; iii, ClCOCOOEt, NEt_3, THF, 1h then CoA-SH, $(Li^+)_3$, predissolved in H_2O.

This is perhaps attributed to the fact that Heck coupling reactions often need a specific set of reaction conditions in order to proceed efficiently. This has been suggested several times[165] and often a range of Heck coupling 'recipes' are screened for optimisation. However, since

we were not concerned with yield and only with the acquisition of the product, no further studies in optimisation was performed.

The next step in the synthesis was the hydrolysis prior to CoA coupling. The same protocol was used as with previous hydrolysis procedures. The rate of conversion was significantly reduced (a reaction time of 72 hours), possibly as a consequence of the high conjugation.

In this instance a mixed anhydride method was employed, using ethyl chloroformate and triethylamine rather than the less reactive CDI, which was ineffective. An acyl chloride on the other hand (*e.g* ethyl chloroformate) is less stabilised by resonance, since the nonbonded pair of electrons on the larger chlorine molecule is unable to delocalise as readily which increases the electrophilicity of the carbanoyl moiety. An alternative reason for the failure of 1,1-carbonyldiimidazole mediated coupling could be due to the stability of the highly conjugated ionic species formed from the delocalisation of a lone pair of the nitrogen in the acyl imidazole (Fig 3.09).

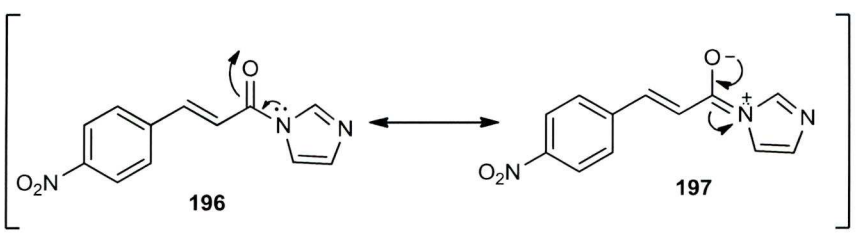


Fig 3.09. Resonance structures of (*E*)-1-(1H-imidazol-1-yl)-3-(4-nitrophenyl)prop-2-en-1-one.

3.4.3 Synthesis of aliphatic chromogen

An additional chromogenic assay substrate was designed. In this substrate *N*-acetylcysteamine (NAC) was used as a truncated analogue of coenzyme A. The proposed enzymatically formed chromophore has a UV max of 263nm. The UV max of coenzyme A is 260nm, which could obscure the product observation. However, *N*-acetylcysteamine does not contain the adenine ring which gives rise to UV absorbance.

Previous studies have shown that NAC thioesters are accepted by other CoA transferases (both type 1 and II). Examples of which are 3-oxoacid CoA-transferase[166] and malonyl-CoA transferase.[167] This gives an additional rationale for the use of *N*-acetylcysteamine. It has also been reported that a number of fatty acyl-CoA ligases (FACL's) display remarkable tolerance

when the CoA group is replaced by *N*-acetylcysteamine.[168] It is known that FACL's contain overlapping substrate specificities,[169] which has also been proposed for AMACR and related β-oxidation enzymes. Furthermore, it was found that varying the chain length did not affect the formation of fatty-acyl-CoA thioesters. These properties draw parallels with AMACR, indicating that NAC thioesters may indeed be tolerated as an alternative to CoA-thioesters. The additional benefits of using *N*-acetylcysteamine rather than coenzyme A are that it is cheaper, much easier to handle and its purification is simpler as the *N*-acetylcysteamine thioesters can be purified by column chromatography. One could also consider using less truncated analogues of CoA such as pantetheine. In this instance, a β,γ-unsaturated thioester would be converted to an α,β-unsaturated thioester giving rise to the chromophore (Fig 3.10).

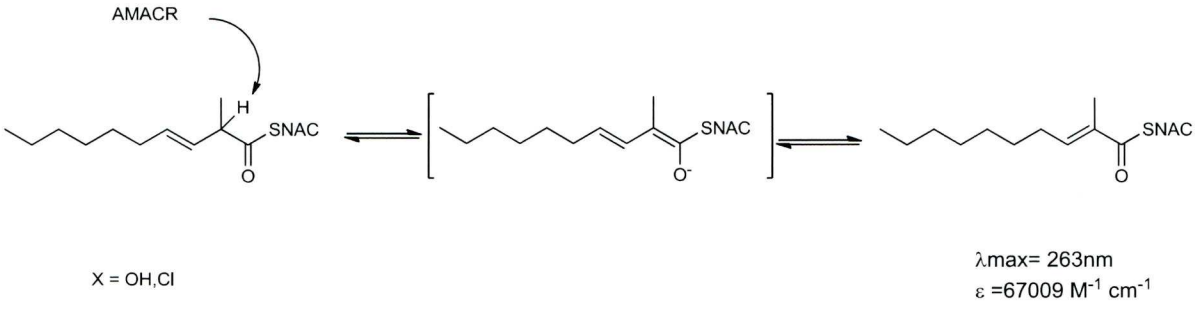


Fig 3.10 Enzymatic formation of the α-β unsaturated aliphatic chromophore.

The synthesis of the chromogenic substrate, (*Z*)-*S*-(2-acetamidoethyl)-2-methyldec-3-enethioate (**72**) began by using (*E*)-ethyl dec-2-enoate (**199**) and methylating in the α-position with the use of DMPU, LDA and methyl iodide. In this instance DMPU is used as a less toxic analogue of HMPU, which is used to coordinate to the lithium atom of the enolate. This helps de-aggregate the enolates, which results in a more reactive species and promotes nucleophilic addition. In doing so, it also helps suppress the formation of (*Z*)-ethyl 4-methyldec-2-enoate (**202**). In this event the reaction yielded (*Z*)-ethyl 2-methyldec-3-enoate (**200**).

It is interesting to note the change of stereo geometry of this transformation. This can be explained by the resulting carbanion that is formed. Experimental evidence for the crotyl anion system (which is analoguous to this aliphatic substrate) demonstrates that the *cis* carbanion is more stable then the *trans* counterpart (Fig 3.11). This can be explained by the possibly greater strain of the CCC bond angle (133°) in the *trans* geometry[170] (**199**) and also by a possible 'aromaticity' that may be present in the *cis* conformation (**198**) by the alignment of the pi orbitals.[171]

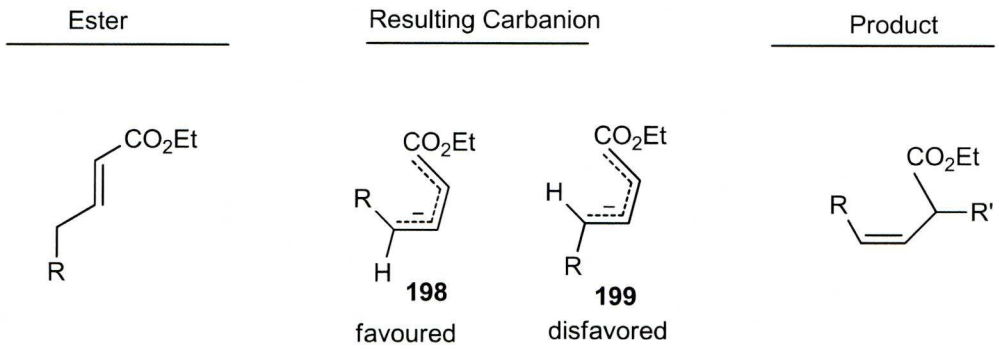


Fig 3.11 Schematic representations of the relative geometries of the favoured/disfavoured ensuing carbanions formed from the (*E*) isomer when exposed to LDA.

The Z isomer was confirmed by measuring the coupling constants of the double bond (Fig 3.12).

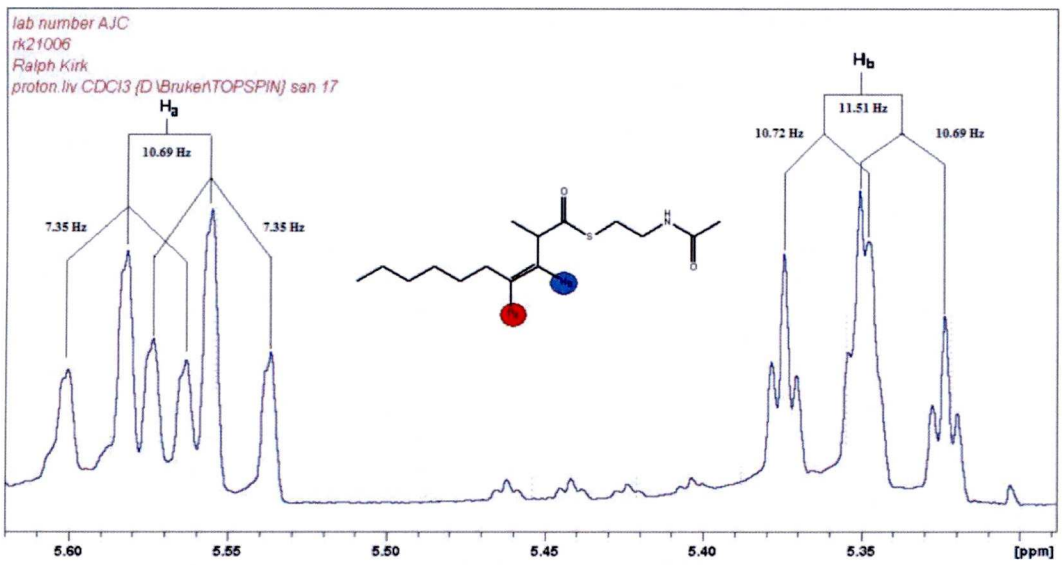


Fig 3.12. An NMR snapshot of (*Z*)-(*S*)-(2-acetamidoethyl)-2-methyldec-3-enethioate (**72**) - highlighting the alkene protons.

Generally *vicinal* coupling constants are larger for *trans* (range: 12–18 Hz, typical: 15 Hz) than for *cis* (range: 0–12 Hz, typical: 8 Hz) alkenes. Taking H_a as an example, (Fig 3.11) the proton is split into an overlapping doublet of triplets. It is split by the adjacent H_b into a doublet (J = 10.69 Hz therefore indicative of Z geometry) which is then further split into a triplet by the adjacent CH_2 protons. H_b is slightly more complicated. What seems to be a triplet of triplets is not the case. It is expected that the proton signal will split into a doublet of

doublets by the adjacent H_a and methine couplings. Splitting by the methine (J = 11.51 Hz) and the vicinal alkene proton (J =10.69 and 10.72 Hz) is observable. This is followed by distant (4 bond) coupling to the alkyl protons adjacent to H_a (J = 1.28 Hz). This was confirmed by correlation spectroscopy (COSY).

The ethyl ester was hydrolysed by refluxing the ester in an aqueous solution of barium hydroxide. The mild base was used to avoid re-conjugation of the double bond. This was then thioesterified with carbonyldiimidazole and *N*-acetylcysteamine (Fig 3.13).

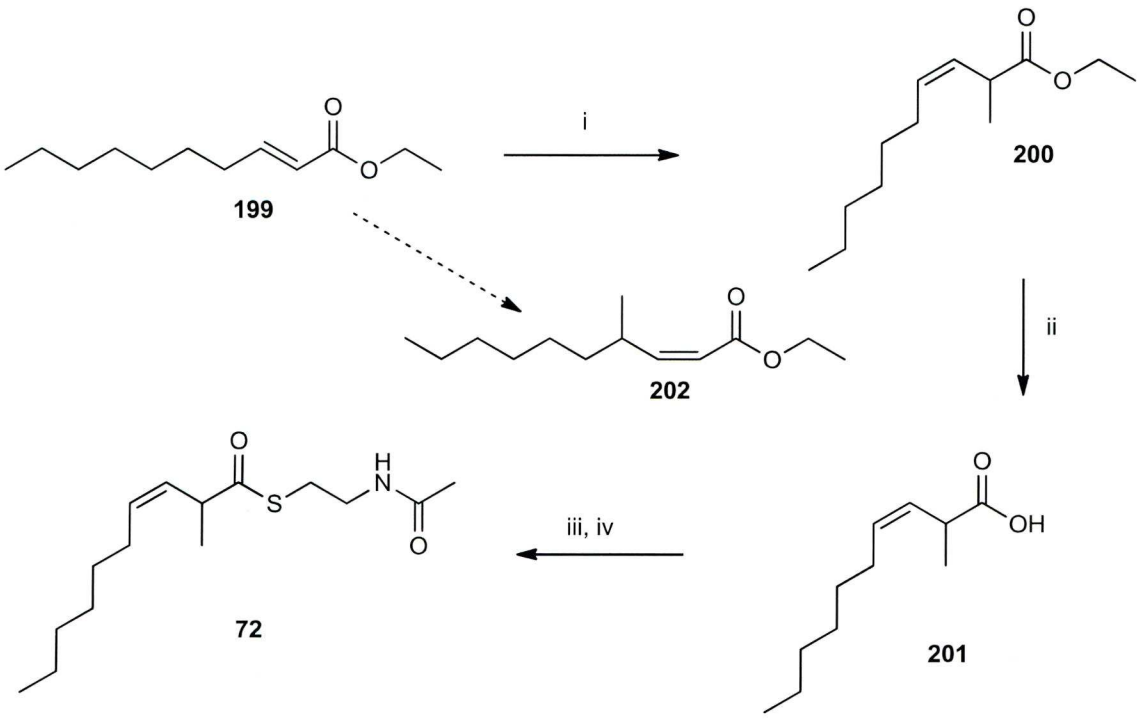


Fig 3.13 *Reagents and conditions*; i, LDA, DMPU, -78°C, 30 mins then (*E*)-ethyl dec-2-enoate for 1h followed by MeI, 2h, -78°C-rt, 45%; ii, $Ba(OH)_2.8(H_2O)$, H_2O, 100°C, ON, 80%; iii, CDI, THF, rt; iv, NAC, THF, rt, 2h 64%.

3.4.4 Synthesis of aliphatic chromophore (**74**)

Synthesis of the conjugated chromophore was attempted using a method analogous to that used for α–trifluoromethyltetradecanoyl-CoA (**49**). Octyl bromide (**203**) was converted into the phosphonium salt (**209**) by refluxing with triphenylphosphine. A Wittig reaction was then performed with methyl pyruvate to yield the α,β-unsaturated ester (**205**) (1:1 mixture of *E* and *Z*) which was then subsequently hydrolysed with sodium hydroxide. It is noteworthy to add that although the stereochemistry of the chromogen and chromophore is different (*Z* and *E/Z* respectively), it is anticipated that the enzyme should tolerate both stereoisomers. We have some confidence of this as the hydrophobic pocket accepts long chains (*cf* same interacting residues as the *E* isomer) and also accepts very bulky steroidal derivatives (*cf* same interacting residues as the *Z* isomer).

Thioesterification was then attempted using CDI and ethyl chloroformate (Fig 3.14). In both cases no thioesterification occurred. Possible due the same reason as described in Section 3.4.2.

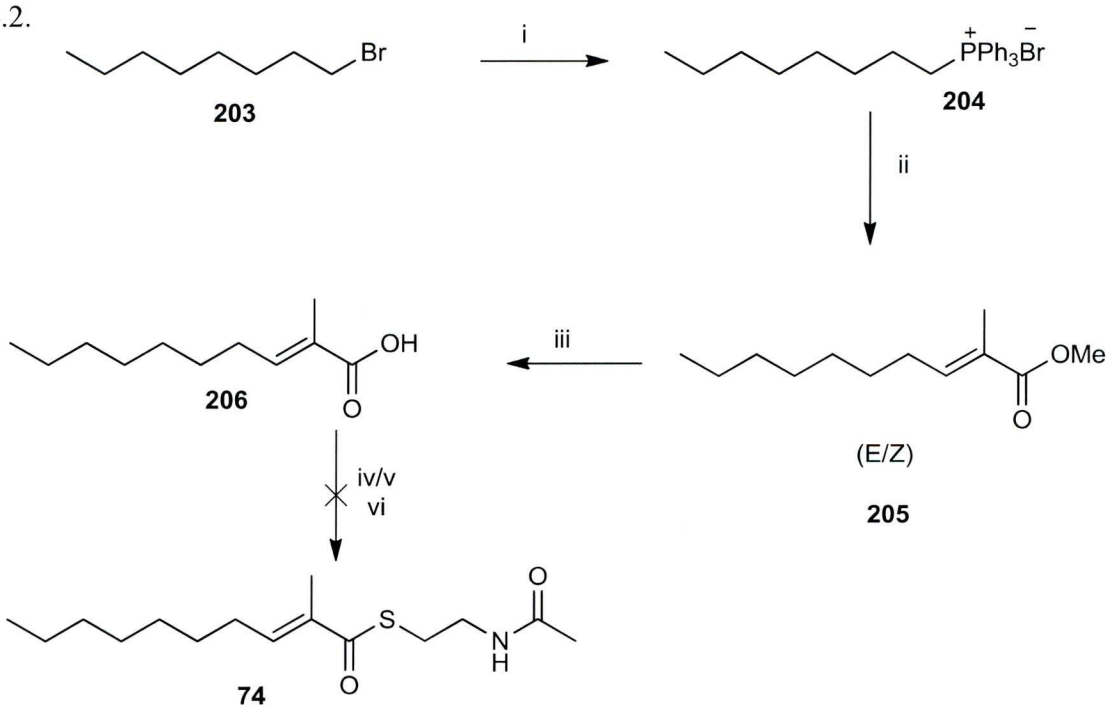


Fig 3.14 *Reagents and conditions*; i, PPh_3, Toluene, reflux 48h, 99%; ii, BuLi, THF, -78°C, $CH_3C(O)CO_2CH_3$, 2h 45%; iii, 5N NaOH MeOH, rt, on, 70%; iv $ClC(O)CO_2Et$, NEt_3, THF, 2h, rt; v, CDI, THF, 4h, rt; vi, NAC, THF, 2h, rt.

In fact, the activated carbonyl intermediates were so stable; a full set of spectra was obtained without hydrolysis. Although the aliphatic chromophore could not be synthesised, we can still identify whether the chromogen is an accepted substrate by monitoring the change in UV absorbance. If it is accepted, then the chromogen should be formed, which will absorb UV light at 307nm.

CHAPTER 4

ASSAY RESULTS

4.1 Initial studies of the racemisation of AMACR.

To initially ascertain the activity of AMACR towards Ibuprofenoyl-CoA (**22**), we took a crude extraction of AMACR from rat liver (extracted by Dr Rob Gibson). This was then incubated with enantiomerically pure (*S*)-Ibuprofenyl-CoA (**22a**) for two hours. Aliquot portions were removed from the reaction mixture at regular intervals, subjected to base hydrolysis (which cleaves the thioester and quenches the enzyme) and extracted into Et_2O. The extent of racemisation was noted by injecting the hydrolysed sample onto a chiral OJ column as described in Section 5.10. (Fig 4.1).

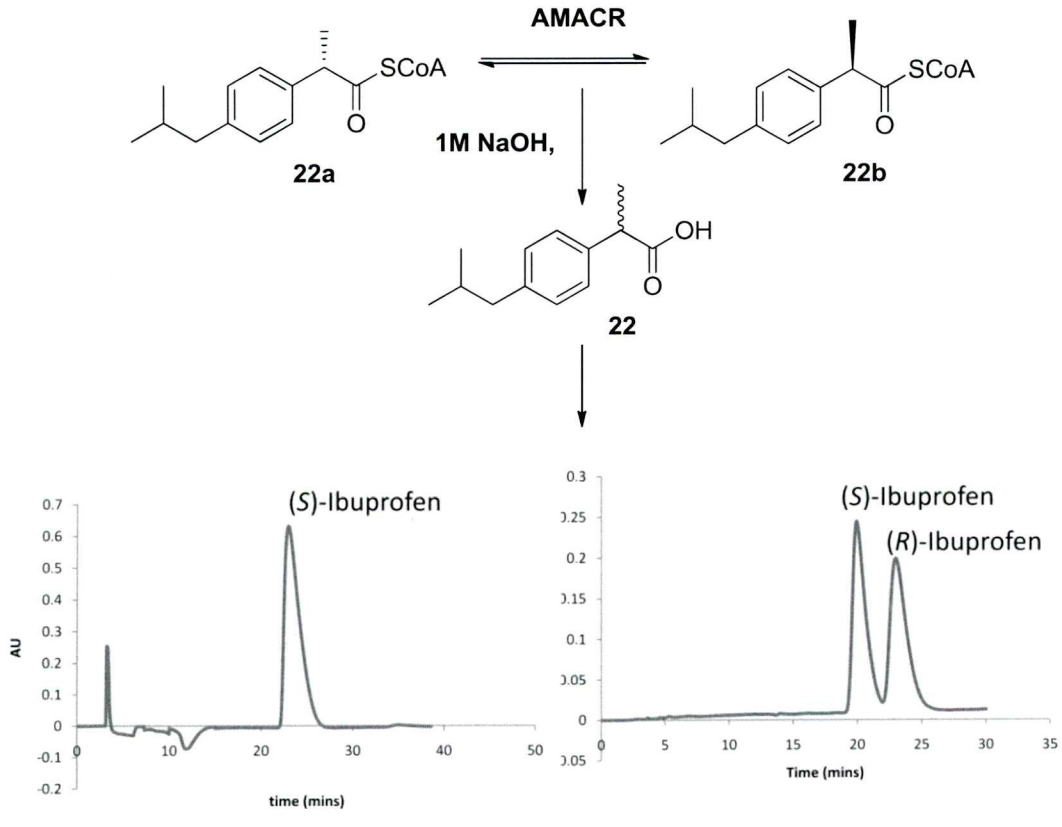


Fig 4.01 First method of analysing the activity of AMACR and relevant HPLC traces. Peaks at 20.0 and 24.6 mins correspond to the (*R*)-enantiomer and (*S*)-enantiomer respectively. For column conditions – see experimental.

4.2 Reverse phase HPLC assay

Although the initial method of assessing the activity of the enzyme *via* injecting the hydrolysed sample onto a chiral OJ column is functional, it is not a practical route for an assay as it requires post incubation chemical modification and lengthy eluting times on the chiral OJ column. In total, each aliquot portion takes approximately 1hour 40 minutes to ascertain the extent of conversion. The use of chiral reverse phase HPLC was then investigated. We found that the use of a chiral AGP column could separate the two enantiomers of Ibuprofenoyl-CoA.[172] This column is made up of an α1-acid glycoprotein as a chiral selector, immobilized on spherical 5μ silica particles. The retention and enantioselectivity is regulated by changes of pH, buffer concentration and organic modifier. It was found that the use of 50mM KH_2PO_4-NaOH buffer (pH 6) containing 4% (v/v) IPA gave adequate separation of the two enantiomers (Fig 4.2). With the crude rat liver extract it was necessary to purify the incubated Ibuprofenoyl-CoA through a C-18 column before loading it onto the chiral AGP column to protect it from degradation. This is an inefficient approach as the length of time to analyse each sample is comparable with the aforementioned use of a chiral OJ column. Ultimately we decided to use MCR (expressed in *E. coli* by Dr Rob Gibson) which enabled us to directly inject the incubated sample onto the column without the risk of decomposing the protein based stationary phase.

A 10% (v/v) solution of TFA was used as a quenching reagent. The use of acetone to precipitate the enzyme was not ideal as the observed peaks of the substrates were dominated by the solvent peak of acetone. An additional obstacle with this method is that the retention time of (*S*)-Ibuprofen (**177a**) is similar with that of (*R*)-Ibuprofenoyl-CoA (**22b**). If hydrolysis occurs, this could result in misinterpretation of the results. Although a possible hindrance, this is rarely observed and can be easily distinguished using a photodiode array detector. Below is an example of the HPLC assaying method and results whereby TFA was used as the quenching reagent (Fig 4.2).

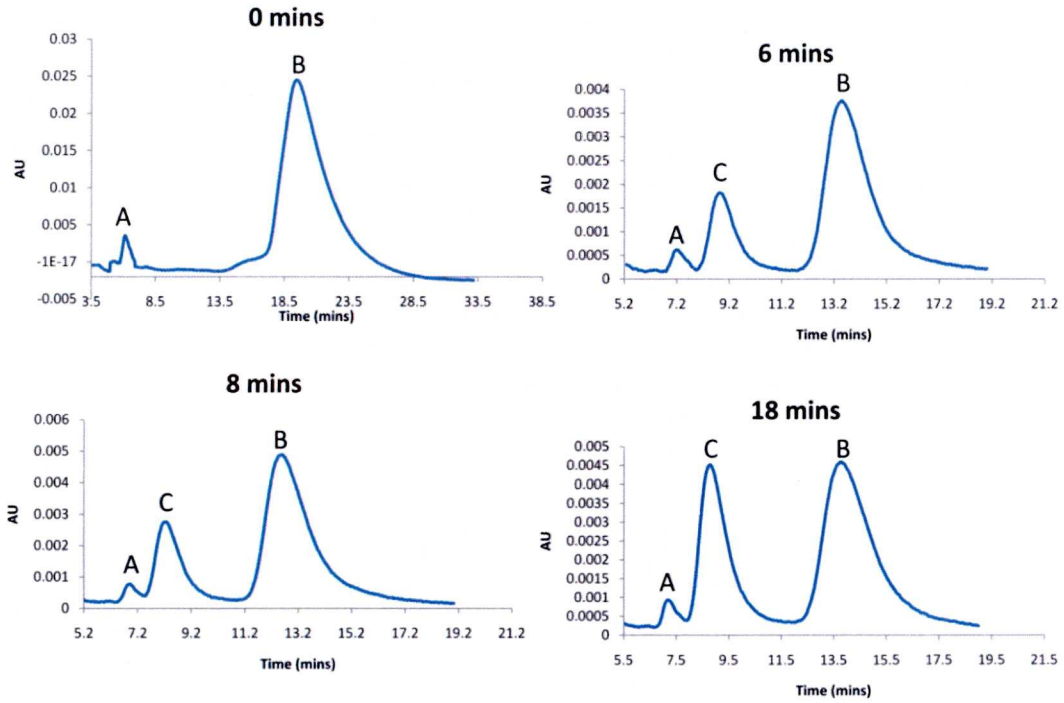


Fig 4.02a. HPLC traces of incubated (*S*)-Ibuprofenoyl-CoA with MCR. Peak A corresponds to residual Ibuprofen. Peak B corresponds to (*S*)-ibuprofenoyl-CoA and peak C corresponds to (*R*)-ibuprofenoyl-CoA.

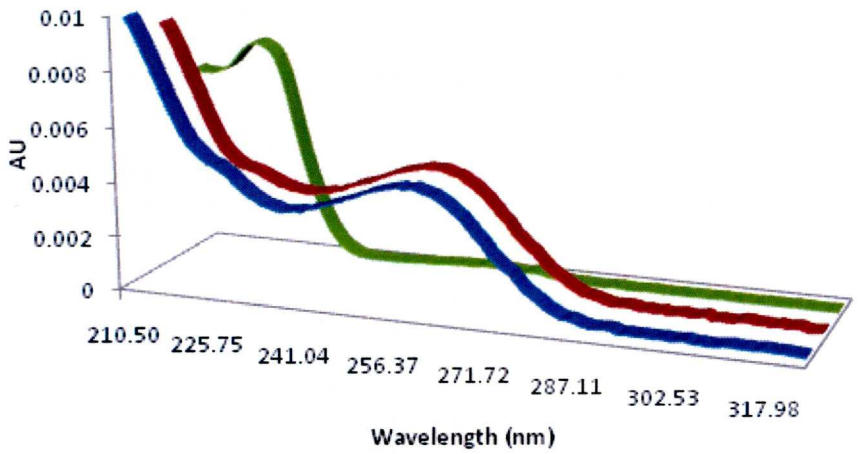


Fig 4.02b. UV absorbances of peak A (green), peak B, (red) and peak C (blue). λmax = 222, 258 and 258nm respectively.

From this data, we began to generate some preliminary enzyme kinetics. Below are two identical runs of 100μM (*S*)-Ibuprofenoyl-CoA with 100ng of MCR in 1ml 10mM MOPS-CH_3CO_2Na buffer, pH 7, run at 37°C (Fig 4.3).

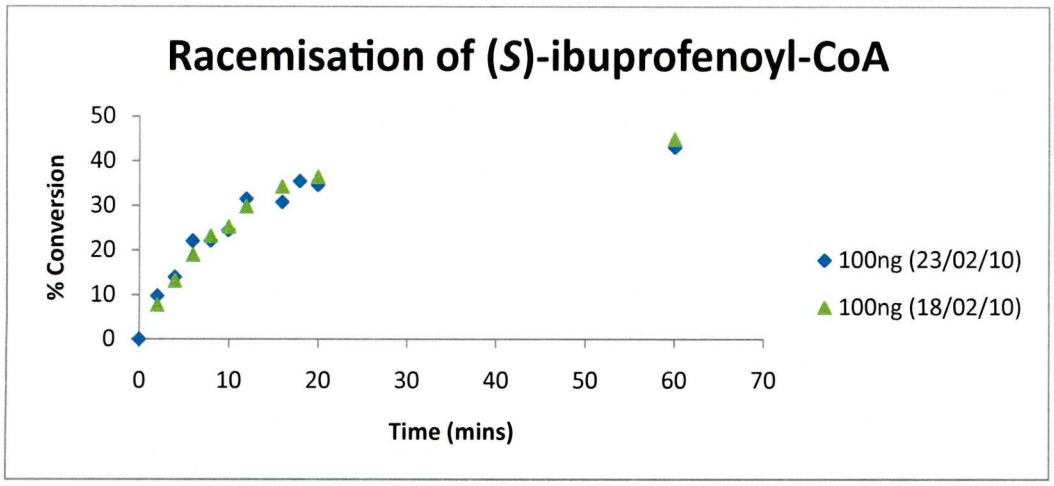


Fig 4.03 Conversion of 100µM (*S*)-Ibuprofenoyl-CoA to its (*R*)-enantiomer with 100ng/ml MCR in MOPS-acetic acid pH 7 buffer performed on two separate occasions.

The enzyme generated reproducible results within a practical time frame (60mins). Next was the examination of enzyme kinetics. This was achieved by maintaining a constant substrate concentration (100µM) and changing the enzyme concentration (Fig 4.4). Due to an increase in active sites available for the substrate to bind, we would expect an increase in enzyme concentration to provide us with a proportionate increase in initial rate of conversion of (*S*)-Ibuprofenoyl-CoA to its racemate.

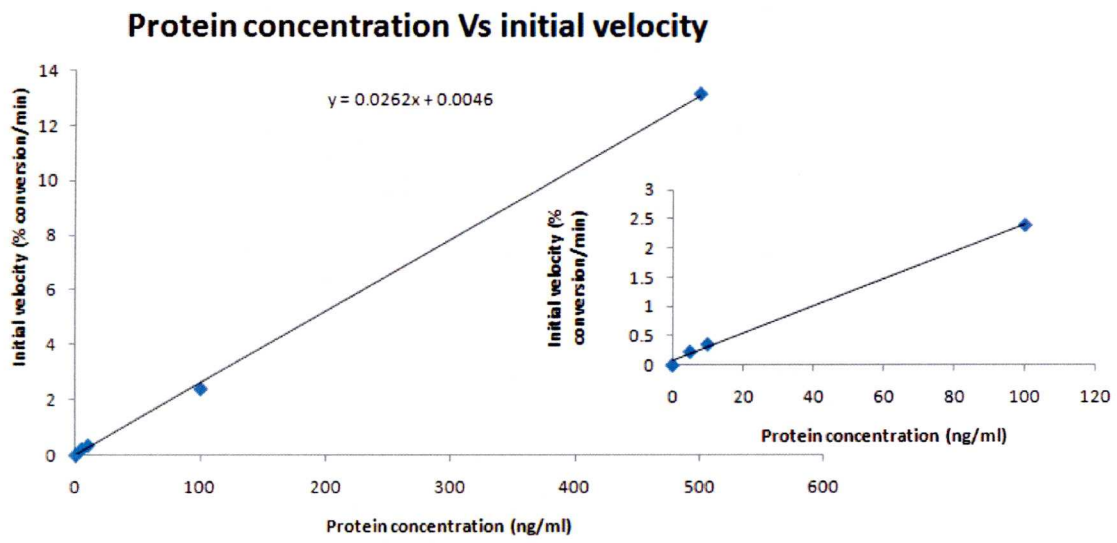


Fig 4.04 Protein concentration of MCR vs initial velocity of racemisation. For raw data see Appendix 3.

It should be noted that the line of best fit intersects the graph at nearly 0.00. This is to be expected for normal enzyme kinetics. We then began to collect data for *Km* and V*max*. This was achieved by varying the concentration of (*S*)-Ibuprofenoyl-CoA and maintaining a constant concentration of enzyme (Fig 4.5). In this case 50ng/ml MCR was used.

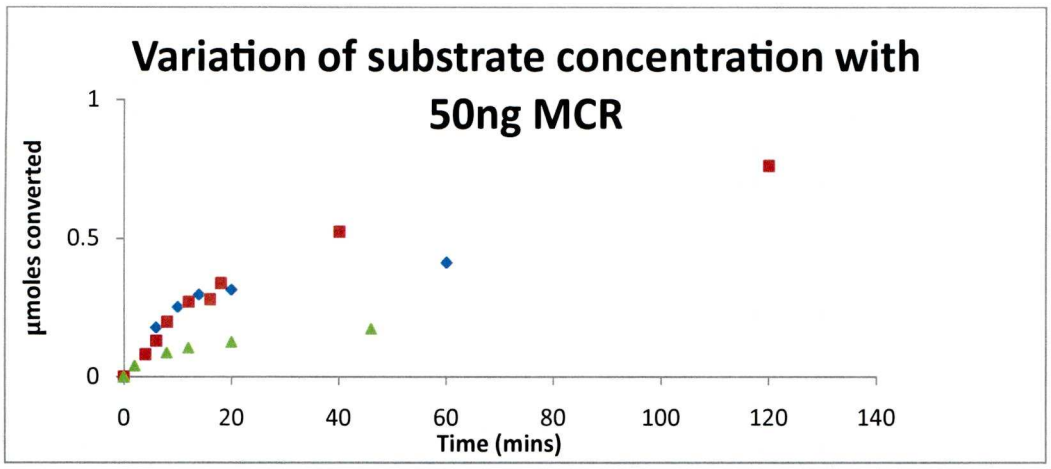


Fig 4.05 The monitoring of racemisation of various (*S*)-Ibuprofenoyl-CoA concentrations with 50ng/ml MCR in pH7 buffer at 37°C. ■ 200µM, ◆ 100µM, =▲ 50µM

V*max* or V_0 represents the maximum rate of enzyme reaction. A plot of initial velocity versus substrate concentration increases in a hyperbolic fashion to approach the characteristic maximum rate, Vmax, at which essentially all enzyme is saturated with substrate (Fig 4.6). *Km* is known as the Michaelis constant, this represents the substrate concentration that results in a reaction rate equal to one-half of V*max*. This is characteristic for each enzyme acting on a given substrate and is a measure of binding affinity.

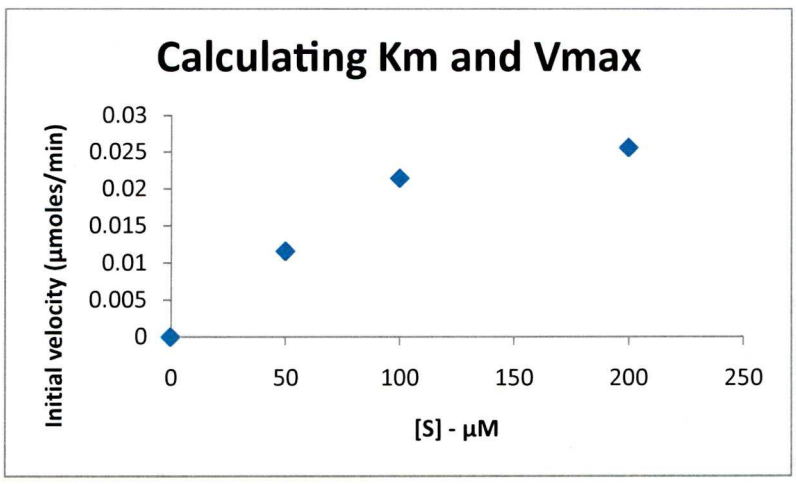


Fig 4.06 Concentration of (*S*)-Ibuprofenoyl-CoA (µM) vs. Initial velocity (µmoles/min).

Using the data from Fig 4.5, the Michaelis-Menten equation (Equation 1, Fig 4.7) and rearranging this to the Lineweaver-Burk equation (Equation 2, Fig 4.7), we can use the raw data from Fig 4.6 to calculate the *Km* and *Vmax* by plotting the double reciprocal plot, 1/V vs 1/[S] (Fig 4.8).

$$V = \frac{Vmax[S]}{Km + [S]} \qquad \text{Equation 1}$$

$$\frac{1}{V} = \frac{\text{Km}}{\text{Vmax}} * \frac{1}{[S]} + \frac{1}{Vmax} \qquad \text{Equation 2}$$

$$Y = m * x + c$$

Fig 4.07 Rearrangement of the Michaelis-Menten equation to produce a linear representation of enzyme kinetics.

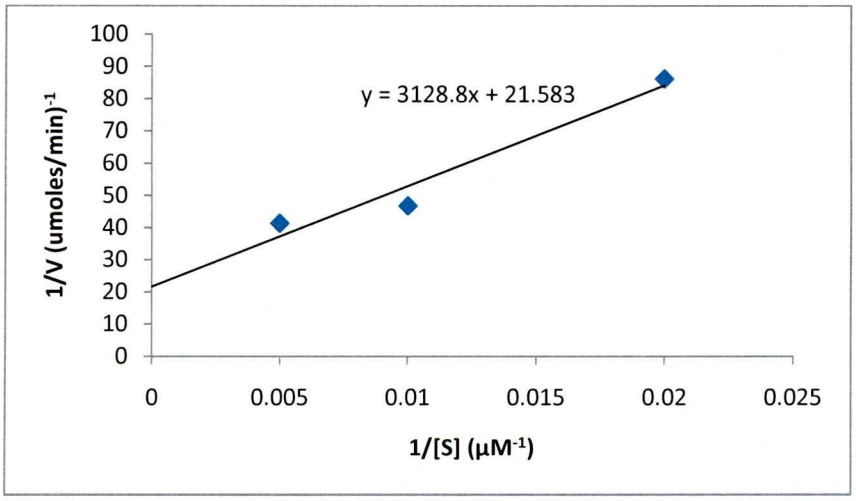


Fig 4.08 1/[S] of (S)-Ibuprofenoyl-CoA (1/µM) vs 1/Velocity (1/µmoles/min)

The values of *Km* and V*max* are 144.97µM and 0.0463µmoles/min respectively. This is in reasonably close accordance with the recent literature values published by Ouazia *et al.*,[173] in which they measure the racemisation of Ibuprofenoyl-CoA by MCR using a circular dichroism (CD)-based assay. This recorded a *Km* for (*S*)-Ibuprofenoyl-CoA of 86+/-6µM. It is interesting to note that this article also confirmed the (slight) preference for MCR to racemise (*R*)-Ibuprofenoyl-CoA with a *Km* of 48+/-5µM.

Unfortunately, we were unable to obtain any more data as the enzyme's activity became unreliable and by the time constraints of this project. Ideally all of these results would be repeated in triplicate and at least one more variation in substrate concentration would be included to obtain a more accurate picture of the enzyme kinetics. Nonetheless, the data obtained thus far is in good agreement with the recently published literature.

4.3 LCMS assay

We also introduced development of an LCMS-based assay as described in the introduction. The retention time of the substrate was two minutes, which meant a very short eluting time per sample. Additionally the peak shape was sharper and the substrate could be detected at lower concentrations. Below is a table outlining the comparisons between the two LC assay approaches.

Variant	Chiral Resolution	Mass/charge resolution
Run time per sample	30 minutes	5 minutes
Lowest detection levels	Approx 0.5 nmoles	0.1-0.25 nmoles
Substrate	Easily synthesised	Longer synthesis
Peak shape	Broad and possible merging with hydrolysed free acid.	Sharper and no possibility of peak merging with free acid.

Table 4.1 Comparisons of the HPLC/LCMS assay systems.

To re-iterate the LCMS approach, enzyme kinetics were to be measured by monitoring the rate in which AMACR de-deuterates α-deutero-ibuprofenoyl-CoA (**67**) and protonates the supposed enzyme bound enolate to form Ibuprofenoyl-CoA (**22**). It was not possible to monitor the rate of de-deuteration/protonation by taking a ratio of the two peaks. This was due to 'cross channelling' between the protonated and the deuterated form - The decrease in peak area of deuterated Ibuprofenoyl-CoA (**67**) did not represent a true reflection of the rate of de-deuteration as the protonated form interfered with the signal. However, this is not the

case *vice versa* which allowed us to monitor the reaction by measuring the peak area increase of protonated Ibuprofenoyl-CoA. The results of incubations with 100μM α-deutero-Ibuprofenoyl-CoA (**67**) and 100μM (*S*)-Ibuprofenoyl-CoA (**22a**) with 50ng MCR at 37°C at pH 7 can be seen below (Fig 4.09):

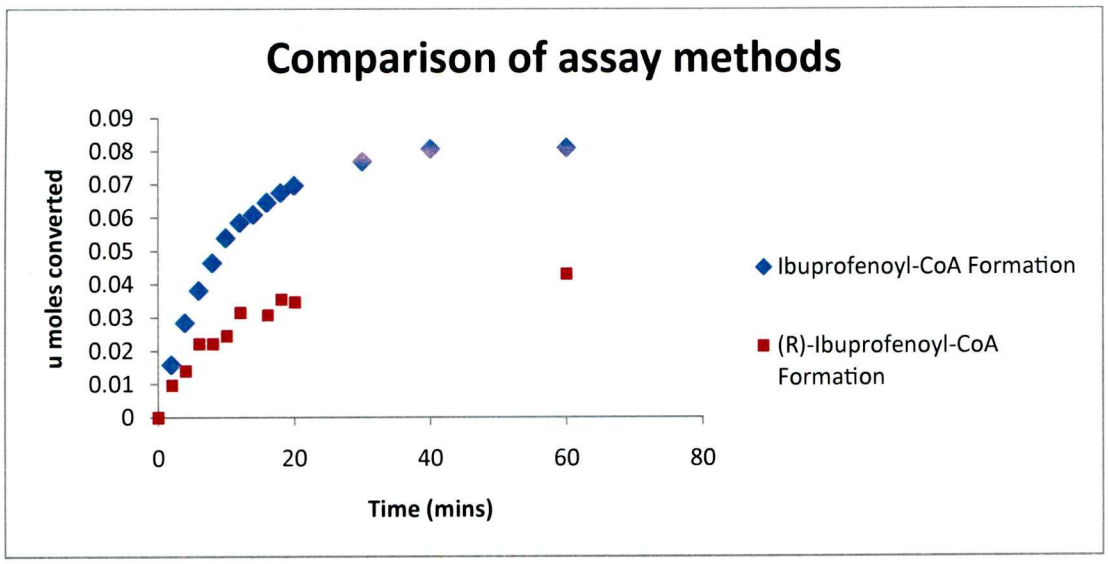


Fig 4.09 Incubation samples of α-deutero-Ibuprofenoyl-CoA (blue) and (*S*)-Ibuprofenoyl-CoA (red) with 100ng MCR.

The initial burst of de-deuteration/protonation is almost double that of the rate of racemisation (with gradients of 0.0047 μmoles/min and 0.0028 μmoles/min respectively – See Appendix 4 for raw data). This can be explained as every time the enantiomerically pure (*S*)-Ibuprofenoyl-CoA (**22a**) enters the active site and is deprotonated, there is a 50/50 chance that is it reprotonated from the same side. Whereas each time α-deutero-Ibuprofenoyl-CoA (**67**) is de-deuterated, the probability that it will re-deuterate is negligible. Each time the deuterium atom is removed from the substrate, it is likely to be lost in the bulk solvent (as explained in Section 1.5.4). Another interesting point about this apparent 'doubling' in rate is that there is no kinetic isotope effect (KIE). If there was a KIE, then the rate of protonation will be significantly slower, owing to the fact the C-D bond requires more energy to break it. This implies that the rate determining step of this reaction is reprotonation. This is in agreement to the proposed mechanism of inhibition of our currently tested inhibitors to date. α-Trifluorotetadecanoyl-CoA (**49**) is postulated to discourage reprotonation by reducing basicity of the enzyme bound enolate (possibly leading to increased binding affinity)

4.4 Results of Chromogenic Assay.

Upon incubating the enzyme with our aliphatic chromogenic substrate (**72**), there was no formation of the chromophore (**74**). We supposed this was due to inability of the enzyme to accept the truncated from of the coenzyme A. There is also the possibility that the chromogen component is not accepted. This was investigated by synthesising racemic and enantiopure NAC thioesters of Ibuprofen (**55/55a**) (Fig 4.10) as the *p*-isobutylbenzene group is a well established accepted side chain for AMACR.

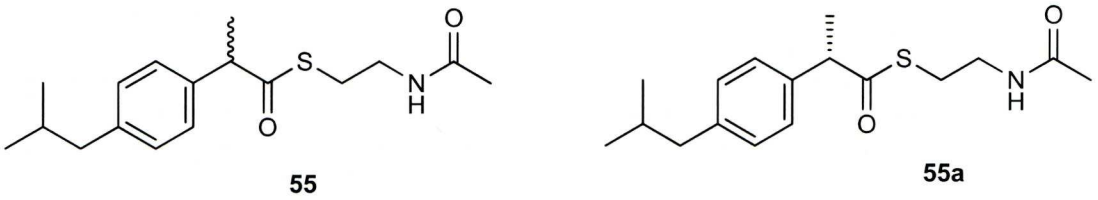


Fig 4.10. Racemic Ibuprofenoyl-SNAC (left) (*S*)-Ibuprofenoyl-SNAC (right).

These enantiomers of NAC thioesters can be separated by chiral chromatography (Fig 4.11).

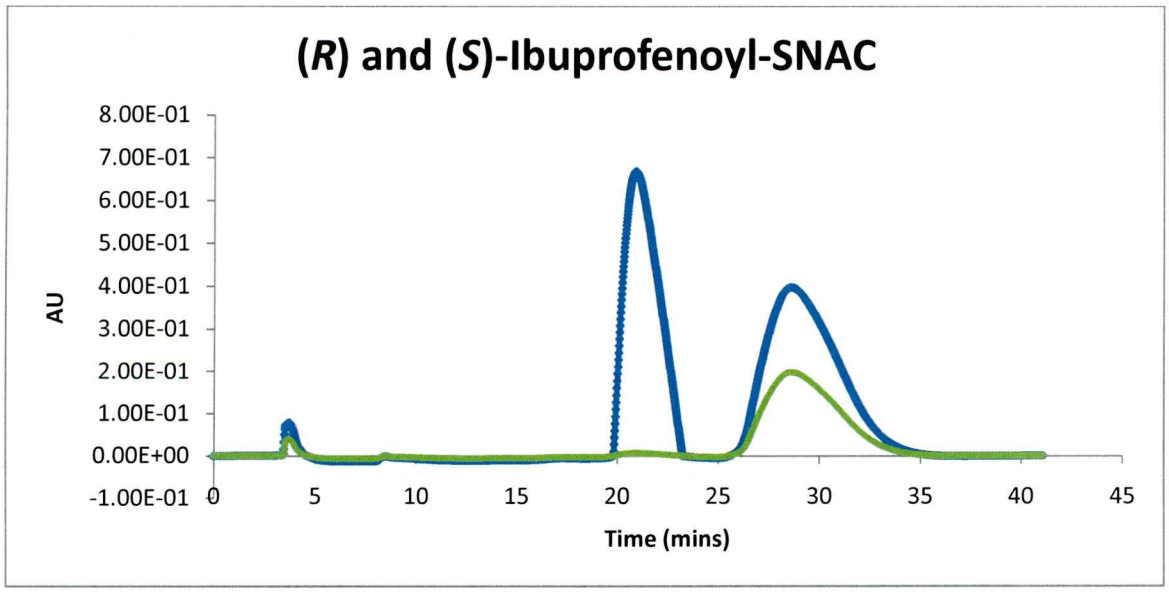


Fig 4.11 HPLCs traces of racemic (blue) and (*S*)-ibuprofenoyl-SNAC (green). The peak at 21.7 corresponds to the (*R*) enantiomer and 29.2 mins corresponds to the (*S*)-enantiomer. Column: Chiral OJ (250mm by 4.6mm). Eluent system: 93.9: 6: 0.1 Hexane: IPA: TFA respectively. 1ml/min.

Incubating (*S*)-Ibuprofenoyl-NAC (**55a**) resulted in no racemisation. Synthesis of the longer pantatheine (**207**) and pantatheine-11-pivolate (**208**) (Fig 4.12) derivatives of Ibuprofen (by Matt Smith) also showed no racemisation.

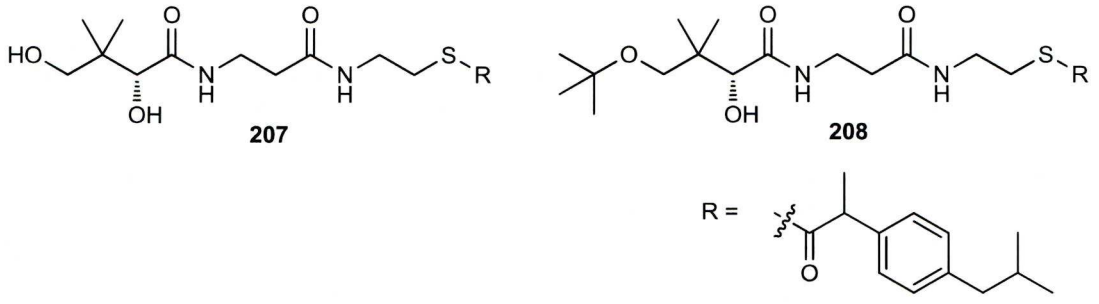


Fig 4.12. Pantatheine (left) and pantatheine-11-pivolate (right).

This indicates that there is an inherent need for the binding of the pyrophosphate and/or adenosine moiety to the enzyme for catalytic activity. From the 3D structure of the enzyme (Fig 4.13), it can be visualised that the adenine ring 'anchors' near the surface of the enzyme, which helps guide the rest of the substrate towards the active site/hydrophobic pocket, where it is further stabilised.

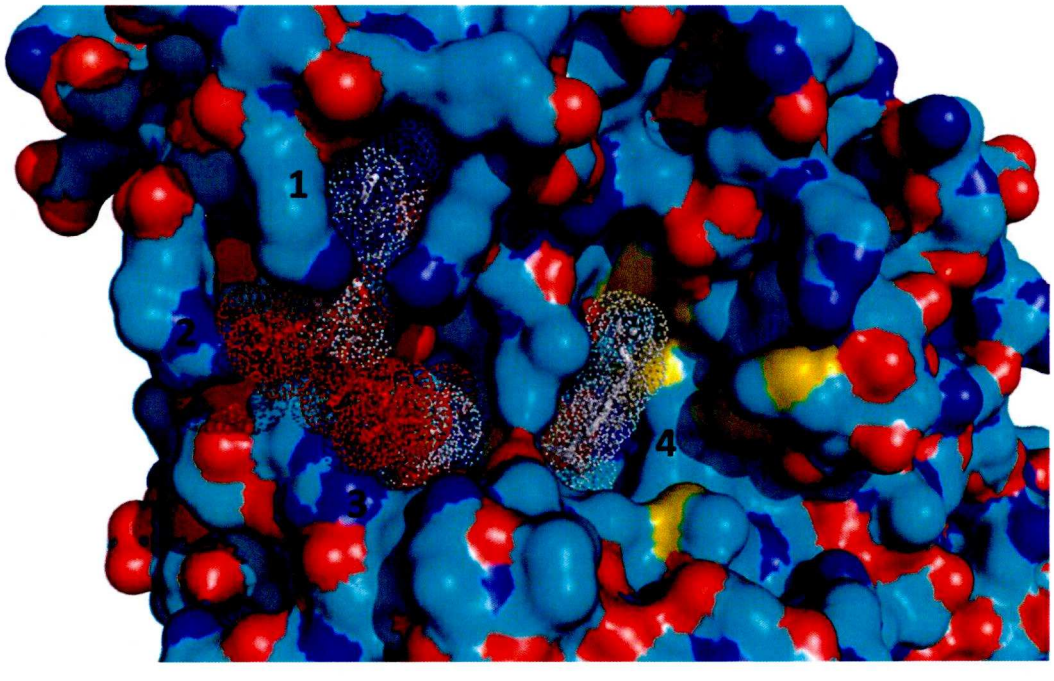


Fig 4.13. Pictorial representation of Ibuprofenoyl-CoA bound to MCR. Pdb file taken from Wierenga *et al.*,[61] 1. Adenine. 2. 1-deoxyribofuranose-3-phosphate 3. Pyrophosphate 4. Ibuprofen.

Additionally, incubation of the aromatic chromogen (**68**) with MCR also yielded no chromophore (**71**). Measuring the UV absorbance of the incubated sample under standard conditions of 37°C and pH 7 for 3 hours did not yield any discernable difference in the UV light absorbed. Refocusing on the β–chloro derivative or perhaps a β–acetate derivative may be a worthwhile endeavour.

Below is an HPLC trace and UV spectrum of the resulting chemically synthesised chromophore (**71**) anticipated (Fig 4.14).

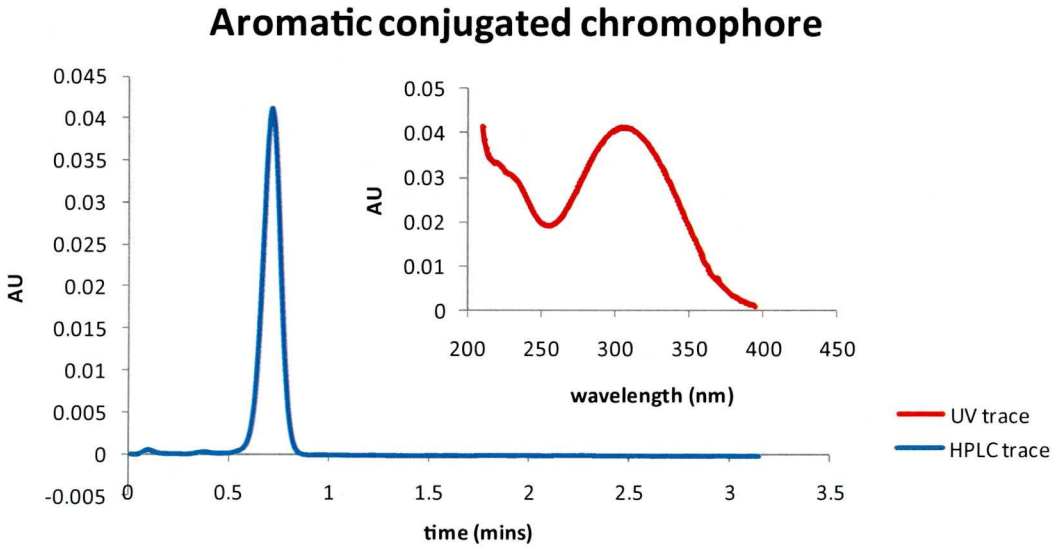


Fig 4.14 HPLC trace and UV absorption of the conjugated chromophore (**71**) expected to form enzymatically.

4.5 Free acid compounds as prodrugs and therapeutic effects

Preliminary preclinical studies have been carried out on two of our potential prodrug inhibitors, α-trifluorotetradecanoic acid (**79**) and β-trifluoroibuprofen (**134**). Professor Chien-Feng and co-orkers from the Department of Pathology, Chi-Mei Medical Center, Taiwan initially screened a range of cancerous cell lines (KEL-FIB, a hepatocellular cancer cell line, OH931, NMHF-1, both of which are myxofibrosarcoma cell lines; and GIST48 and GIST882 which are gastrointestinal stromal cell lines) for expression of AMACRIA, AMACRIIA and PGK (phosphoglycerate kinase – a housekeeping gene[174]) Of these, the most prevalent expression of AMACR was found in GIST48 and GIST882 (Fig 4.15).

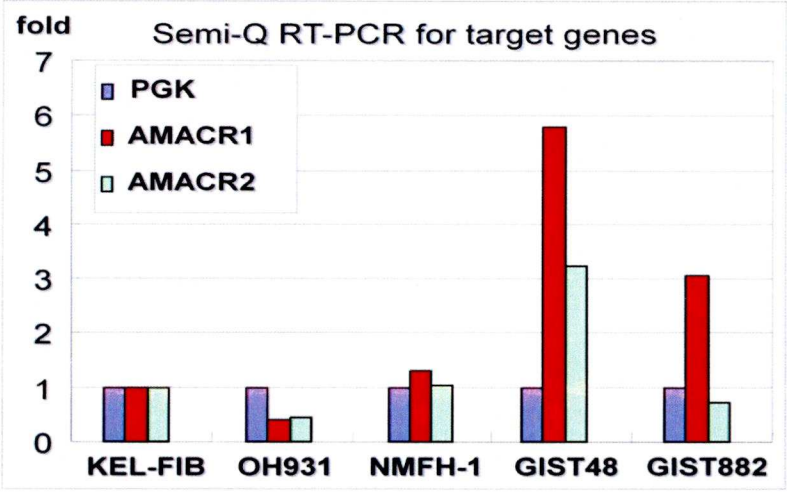


Fig 4.15 AMACR expression in a range of cancers.

The cytotoxic effect of α-trifluorotetradecanoic acid (**79**) was then tested on GIST882 and was found to prove effective despite needing a high concentration (Fig 4.16).

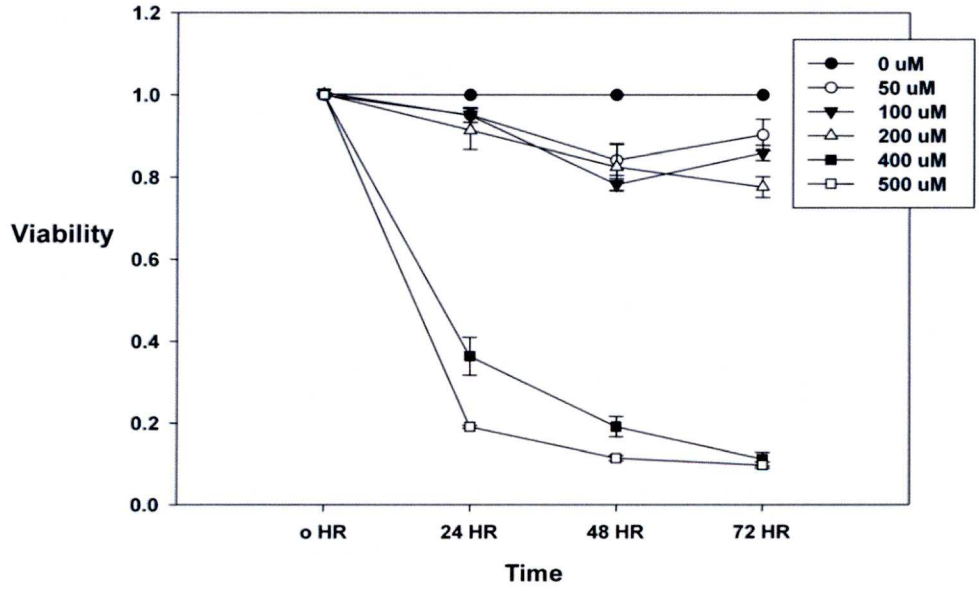
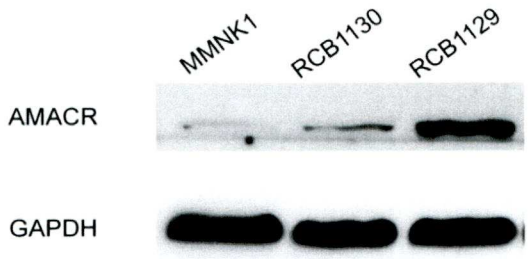


Fig 4.16 Cell viability vs. Time at various concentrations of α-trifluorotetradecanoic acid (**79**).

Next, the two potential prodrugs were tested for toxicity in *M. musculus*. It was revealed that α–trifluorotetradecanoic acid (**79**) had marked toxicity at a concentration of 200 μM. However, β-trifluoroibuprofen (**134**) did not cause any significant acute toxicity even at 800-1600mg/kg (administrated by I.P, three times a week, a total course of two weeks). This was then followed by testing β-trifluoroibuprofen (**134**) on three different gall bladder carcinoma cell types, MMNK1 (an immortalized normal cholangiocyte), RCB1130 and RCB1129,

(biliary tract adenocarcinoma cell lines). MMNK-1 has no expression of AMACR, RCB1130 has some expression of AMACR and RCB1129 has an overexpression of the enzyme (Fig 4.17).



MMNK-1: immortalized normal cholangiocyte
RCB1129 & RCB1130: gallbladder carcinoma cells

Fig 4.17 AMACR and GAPDH (a housekeeping gene used in comparisons of gene expression data[175]) expression in a range of gall bladder cancers.

Incubating β-trifluoroibuprofen (**134**) in these cancerous cell lines demonstrated cancer specific cytotoxicity as follows:

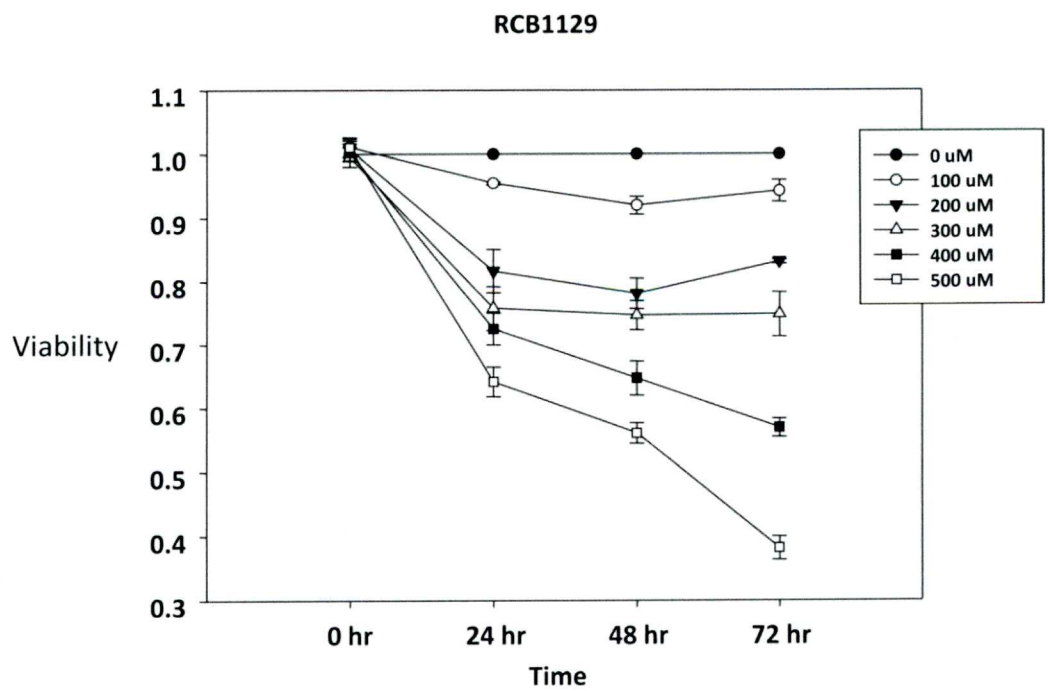


Fig 4.18 Cell viability vs. time for various concentrations of β-trifluoroibuprofen (**134**) against the gall bladder cell line RCB1129.

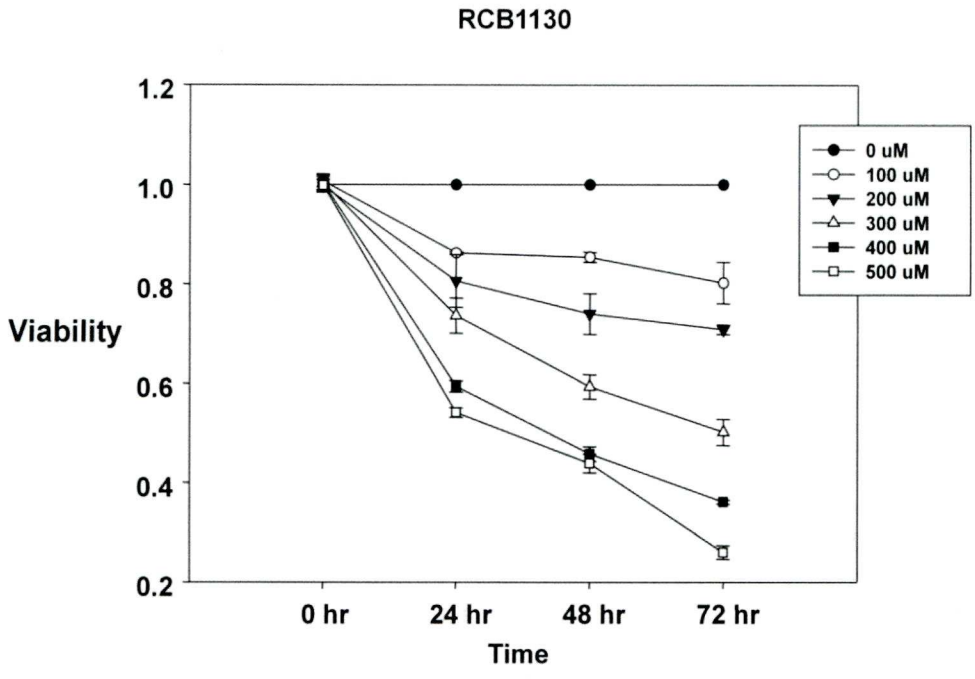


Fig 4.19 Cell viability vs. time of various concentrations of β-trifluoroibuprofen (**134**) against the gall bladder cell line RCB1130.

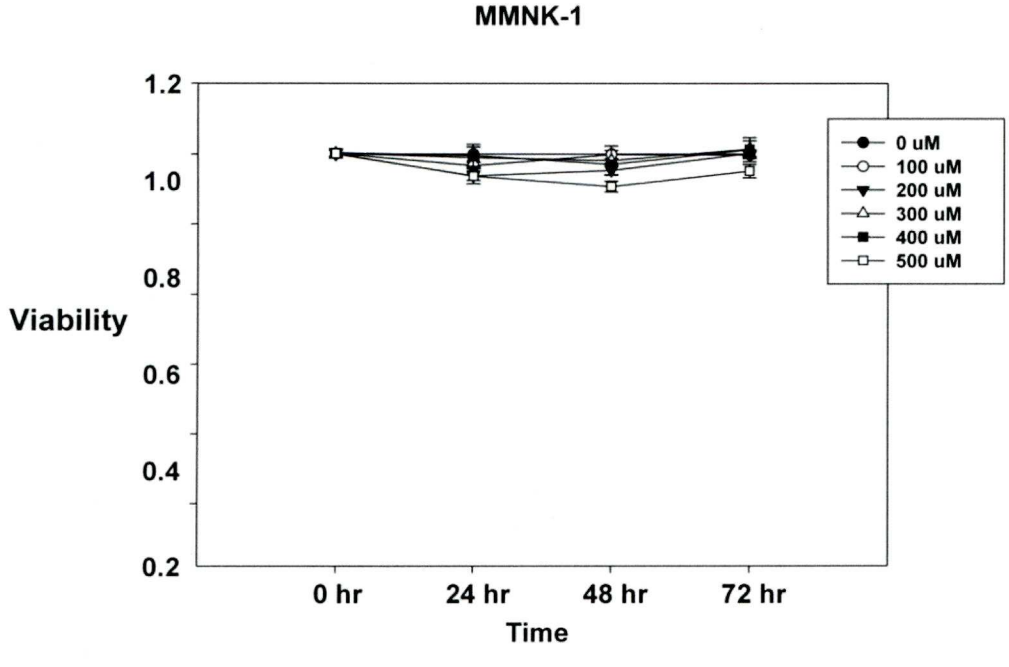


Fig 4.20 Cell viability vs. time of various concentrations of β-trifluoroibuprofen (**134**) against the gall bladder cell line MMNK-1.

It is very encouraging to note that cells which have no expression of AMACR (MMNK-1) are impervious to the potential therapeutic activity of β-trifluoroibuprofen (**134**), whereas RCB1130 and RCB1129 cell lines (which expresses AMACR) have a significant loss of cell

viability. It is however noteworthy to add that the concentration required for significant reduction of cell viability is high. This may be attributed to the fact that *in vivo* thioesterification is slow, due to the substrate being unnatural for the *acyl-CoA synthetase*. It is also worthwhile to note that β-trifluoroibuprofen may act as a COX-1/2 inhibitor,[142] which may alter prostaglandin biosynthesis which can affect hormone regulation and cell growth, producing irregularities in the results.

4.6 Future work

4.6.1 Assay Development and Inhibition

In this work, a range of potential inhibitors and prodrugs have been synthesised. Of these inhibitors two have been tested in preclinical studies as anticancer agents (α-trifluoromethyl tetradecanoic acid (**79**) and β-trifluroibuprofen (**134**)), the latter of which has given some promising results. Nonetheless, there is no categorical proof that the latter works by inhibiting AMACR. It is important that the efficacies of these potential inhibitors are tested against AMACR. From the assay development discussed earlier in this Chapter, the next logical step in this project would be to use the LCMS based assay to test the range of inhibitors that have been synthesised. We can then progress in determining which of these inhibitors offers the best chance as a potential lead for a therapeutic agent.

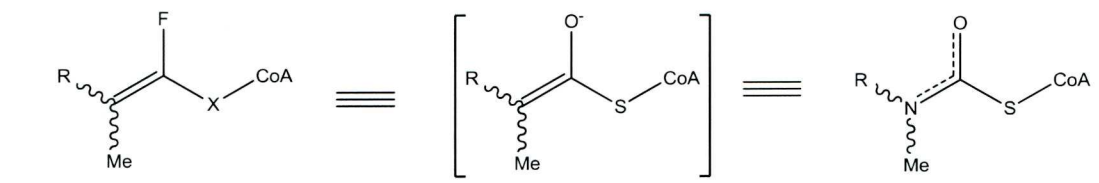
4.6.2 Pro drug strategy and *in vitro* testing.

The distinct advantage of using Ibuprofen derivatives as opposed to the previously studied aliphatic derivates is their increased bioavailability. Nonetheless, the use of CoA thioesters as drug molecules is not a good idea as they are not bioavailable, susceptible to thiolytic cleavage and can possibly interact with other CoA utilizing enzymes. It is unfortunate that the truncated forms of known CoA ester substrates of AMACR have no enzymatic turnover. Therefore with any designed inhibitor, we must consider the fact that only CoA thioesters are accepted. A pro-drug strategy, whereby the proposed inhibitors are in their carboxylic acid form is an attractive approach. This would take advantage of the CoA esterification *in vivo*. To ascertain the likelihood of *in vivo* thioesterification, it would be worthwhile to obtain a relevant CoA synthatase (*e.g long chain fatty acyl-CoA synthetase*) and test whether or not the chosen drug molecule can be CoA esterfied *in vitro*. This data has already been ascertained for some acid precursors, namely compounds (**52**), (**54**) and (**79**) in which all

three were successfully thioesterified.[116] This is encouragingly indicative that other fluorinated derivatives of known substrates of AMACR would also be activated *in vivo*.

4.6.3 Enolate mimics

As a further addition to our arsenal of potential enzyme inhibitors, fluoroalkenes (**209**) and thiocarbamates (**210**), designed as isoelectronic mimics of the assumed substrate enolate anion, may show inhibition of AMACR (Fig 4.21). It is believed that since the enzyme may stabilise the enolate intermediate derivation from natural substrates, enolate mimics may bind strongly to the active site. These compounds are being prepared by a co-worker within the group.



R = $C_{12}H_{25}$, THC or *p*-ibu benzene
X = CH_2, S
209

presumed CoA enolate
stabilised by AMACR

210

Fig 4.21 Proposed enolate mimics as potential inhibitors of AMACR.

4.6.4 Ketone analogues

To overcome the tendency for *in vivo* thiolytic cleavage of thioesters, introducing a CH_2 linker may be a useful strategy. This effectively turns the thioester into a ketone, which will presumably be more biologically stable (Fig 4.22). The α-protons of ketones are slightly less acidic then their thioester analogues, which may alter the binding affinity. However, this strategy has been successfully employed with other CoA utilising enzymes such as *citrate synthase*, *acyl-CoA dehydrogenase* and *palmitoyl transferase*[176] whereby the CoA-thioethers are competitive inhibitors *vs* their CoA-thioester counterparts. As the structural change is small (with the addition of one carbon atom), their structures are ideally suited as stable mechanistic probes for AMACR, involving X-ray structural and kinetic studies. Work is underway in the group to prepare these compounds.

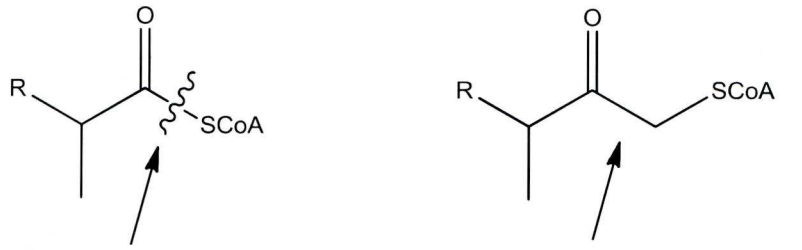


Fig 4.22 Metabolism comparison of thioesters and thioethers.

4.7 X-ray crystal structure of mitochondrial synthetic thiolase.

In an additional study carried out on the crystal structure of cytosolic thiolase by Rik Wierenga *et al.,* the reaction mechanism was endeavoured to be realised. Thiolase is an important enzyme for the synthesis of cholesterol and inhibitors of which are expected to be of medical relevance. Slowing down the synthesis of cholesterol but not completely preventing cholesterol synthesis is particulary important for the medication of children. The synthesis of cholesterol in the brain is important for the maturation of the brain tissue. There are currently no structures available of bound enolised substrates and the reaction mechanism of thiolases are poorly understood. The best studied enzyme in this respect is citrate synthase, but there are no structures available of transition state complexes. A natural substrate of cytosolic thiolase is 2-methylacetoacetyl-CoA. The proposed reaction cycle degrades this to form propanoyl-CoA (Fig 4.23).

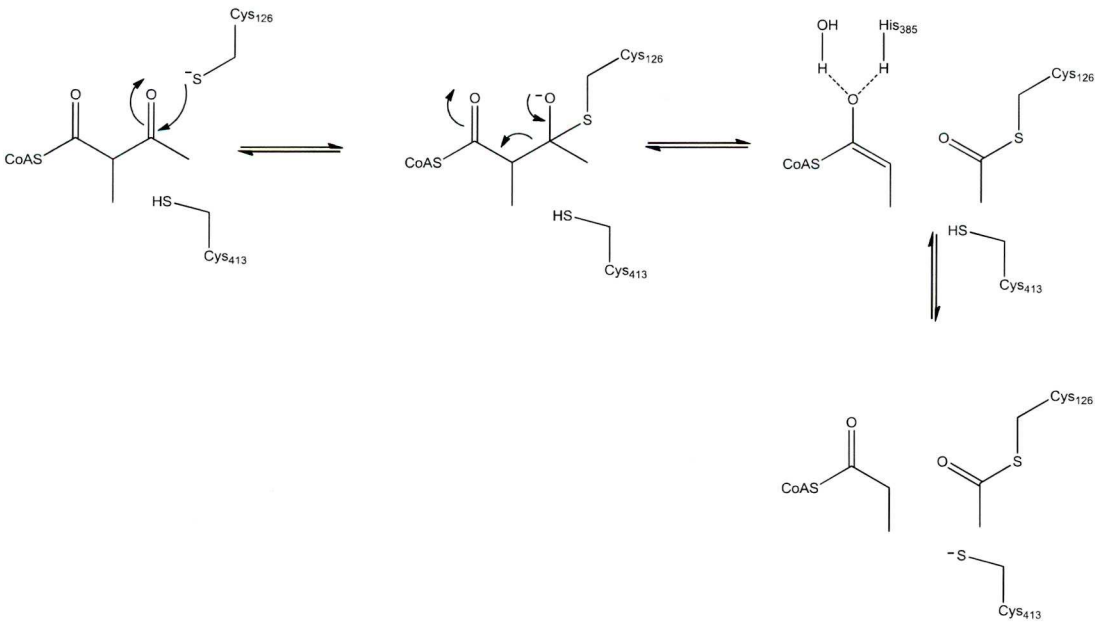


Fig 4.23 Proposed mechanism of mitochondrial synthetic thiolase.

Within the active site of the thiolase there are two oxyanion holes, the first of which stabilises the enolate oxyanion and the second oxyanion hole stabilises the tetrahedral oxyanion. Incorporation of an α–trifluoromethyl group (α-trifluoropropionyl-CoA, **211**) may facilitate a transition state analogue in which the enolate form is stabilised upon binding. A ligand bound X-ray crystal structure could thus be obtained to help verify the reaction mechanism. This could then also give insights into possible potential inhibitors.

Synthesis of this thioester was challenging. Trifluoropropionic acid has propensity to polymerise once activated with ethyl chloroformate or CDI. However, this was circumventented by carefully removing any excess CDI after formation of the activated acyl imidozolium intermediate by washing the reaction medium with water. This was then subject to thioestification with coenzyme A to yield the desired product (**211**) (see appendix 2). Compound (**211**) has been soaked in crystals of cytosolic thiolase and is continuing to be used by the Weirenga group in an ongoing investigation as to how it binds to the enzyme.

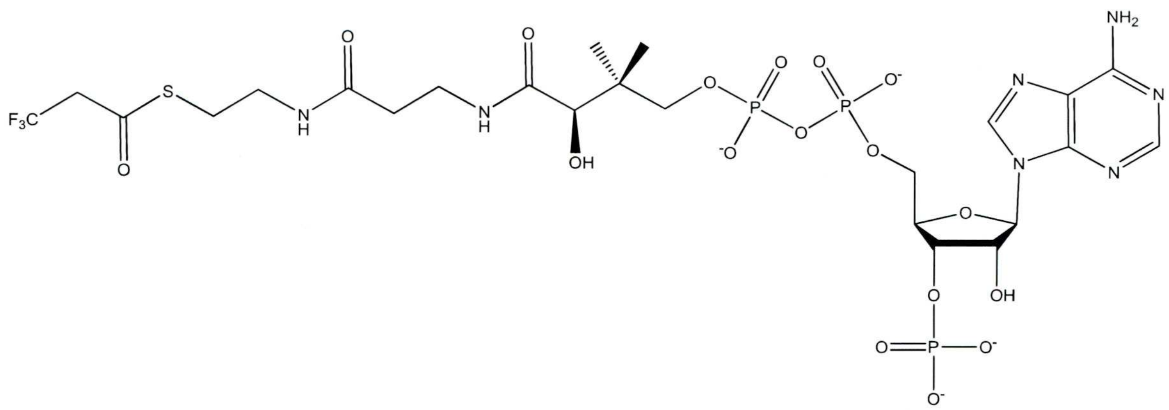


Fig 4.24 Trifluoropropionyl-CoA (**211**).

CHAPTER 5

EXPERIMENTAL

5: REAGENTS AND TECHNIQUES

5.1 Analytical thin layer chromatography (tlc)

Analytical thin layer chromatography was performed on 2 x 5cm aluminium sheets, pre-coated with Merck Kieselgel 60 and detection by UV 254nm light. The developed chromatograms were visualised with a UV lamp. Plates were then treated with potassium permanganate dip and developed with a heat gun.

5.2 Flash column chromatography

The required quantity of Silica (Merck 9385 Kieselgel 60) was made into slurry with the desired eluent and then applied to the column over a thin bed of sand or a sintered glass frit. The crude material was the applied to the column either dissolved in a small amount of the eluent or absorbed onto silica and then the column was eluted with further solvent of increasing polarity. Fractions of 5-100ml were collected and analysed by tlc.

5.3 Mass Spectrometry

Mass spectrometry analyses (ES+, ES-, EI+) were performed on a Trio1000 spectrometer and run by the mass spectrometry service within the University of Liverpool. Where chemical ionisations (CI) were used, ammonia was used as the carrier gas. For all non-trivial compounds high resolution mass spectrometry was performed. For trivial/previously published compounds nominal mass spectrometry sufficed.

5.4 NMR & IR

^{1}H, ^{13}C, ^{19}F, COSY and HMQC NMR spectra were recorded on a Bruker AMX-400 machine. Coupling constants (*J*-values) are reported in Hz. Chemical shifts are given in ppm, downfield from an internal standard of tetramethylsilane (TMS).

IR spectra were recorded as neat samples or diluted with DCM (having ran a background DCM spectra) using a Jasco 4200 FT-IR spectrometer in the range 4000-600cm^{-1}, previously washed with ethanol.

5.5 pH measurements

pH measurements were taken using a Metrohm 691 pH meter. This was calibrated prior to use with buffers purchased from Thermo Russell (pH 4.00, 7.00 and 10.00 ±0.01 at 25°C)

5.6 High pressure liquid chromatography

5.6.1 Analysis of racemisation

Analytical reverse phase HPLC was performed on a Waters 2695 separations module equipped with a Waters 996 photo diode array detector. Separations were carried out using a Chiral AGP, 150mm x 4.0mm column packed with α_1-acid glycoprotein (AGP) as the chiral selector immobilized on spherical 5 μm silica particles. An isocratic gradient using 96% (v/v) 50mM KH_2PO_4-NaOH (pH 6) and 4% (v/v) MeCN with a flow rate of 0.7ml/min was used

5.6.2 Enantiomeric resolution of acid/ester precursors

Analytical normal phase HPLC was performed the same system as 5.5.1. Separations were carried out using a Chiral OJ column. 250mm x 4.6mm column packed with Cellulose tris (4-methylbenzoate) coated on 10μm silica-gel. An isocratic gradient using 98.9% (v/v) Hexane, 1% (v/v) IPA and 0.1% (v/v) TFA with a flow rate of 1ml/min was used unless otherwise stated.

5.6.3 Analysis and purification of substrates/inhibitors

Analytical reverse phase HPLC was performed on a Gilson separations equipped with 306 pumps and a Gilson 118 UV detector. Separations were carried out using a Knauer C-18, 5μ, 250mm x 4.6mm column packed with 5μm eurosphere 100-5 silica. An isocratic gradient using 66% (v/v) 10mM NH_4OAc-CH_3CO_2Na, (pH 6) and 34% (v/v) MeCN with a flow rate of 1ml/min was used unless otherwise stated.

Preparative RP-HPLC was performed on the same Gilson system using a Knauer C-18 5μ, 250mm x 20mm column packed with 5μm eurosphere 100-5 silica. An isocratic gradient using 66% (v/v) 10mM NH_4OAc-CH_3CO_2Na, (pH 6) and 34% (v/v) MeCN with a flow rate of 10ml/min was used unless otherwise stated.

5.7 Liquid chromatography mass spectrometry

Aliquots of the quenched incubated sample (3μL) were chromatographed at room temperature on a Phenomenex Phenyl-hexyl column (150 x 4mm) fitted with a phenyl-hexyl guard column by isocratic gradient elution with 70% (v/v) 10mM NH_4OAc-CH_3CO_2Na buffer, pH 6/ 30% (v/v) MeCN for 10 mins with a flow rate of 0.8ml/min. The flow rate of eluate to the LC-MS interface was *ca.* 160μL/min. An Applied Biosystems/MDS Sciex API 2000 mass spectrometer was operated with a TurboIonSpray electrospray source. The interface temperature was 400°C; electrospray capillary voltage, 5.0kV; heater gas (Gas2) setting 75. The instrument was set up for scanning between the ranges of 956.5 (161.1)-957.5 (162.1) with a scan time of 5s. Instrument management and data processing were accomplished through analyst 1.4 software.

5.8 UV Spectroscopy

The UV absorbances of stock solutions of CoA thioesters were measured using a PerkinElmer Lambda Bio + diode array detector in quartz curvettes of 1cm pathlength. UV absorbance measurements were made by serial dilutions of the stock thioester solution until a reading of 0.3-3 was obtained.

The UV absorbance of the incubated sample of compound (**73**) with MCR was measured using a PerkinElmer Lambda 25 UV/VIS spectrometer with a Peltier water system and temperature programmer.

5.9 Solvents

Analytical grade solvents were used for reactions involving aqueous media. All solvents were distilled and kept over 3Å molecular sieves prior to use. Purified / dried solvents were obtained as follows:

Dichloromethane (DCM)- Distilled from calcium hydride before use.

***N,N*-Dimethylformamide (DMF)**- Sureseal™ anhydrous solvent purchased from Aldrich or distilled from calcium hydride before use.

Methanol (MeOH)- Distilled from a magnesium and iodine still for 2-3 hours under argon atmosphere before use.

Tetrahydrofuran (THF)- Dried from sodium and benzophenone until purple colouration persisted and then distilled under atmospheric pressure.

5.10 Reagents

CoA-SH, $(Li^+)_3$ was purchased from Larodan. All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

5.11 Incubation technique

Example 1: 100μM (*S*)-ibuprofenoyl-CoA (**22a**) with 50ng MCR in 1ml 10mM MOPS-CH_3CO_2Na buffer (pH 7).

A stock solution of MCR in a mixture of 10mM MOPS-CH_3CO_2Na buffer (pH 7) and glycerol (1:1) was supplied by Dr Rob Gibson, and stored in a freezer at -20°C. From the concentration given (4.15mg/ml), the mixture was warmed to room temperature and 12μL of which was mixed with 965μL 10mM MOPS-CH_3CO_2Na buffer, pH 7 (made up of 97μL 100mM MOPS-CH_3CO_2Na buffer solution and 869μL H_2O). A separate buffered solution of ibuprofenoyl-CoA was made using the same buffer as stated and the concentration was accurately calculated with use of the extinction coefficient of CoA (λmax of 260nm - extinction coefficient of $15800M^{-1}cm^{-1}$ [121]) to be 4.35mM. Both samples were placed in an incubator at 37°C, whilst being stirred at 180 rpm for 10 minutes, after which time 23μL of the substrate sample was injected into the enzyme solution. At regular intervals aliquot portions of the incubated sample were removed (50μL) and injected into a vial containing an aqueous 10% (v/v) TFA solution (5μL). The samples were well mixed (by removing the entire contents of the vial (10μL) and re-introducing the solution into the same vial several times with use of a micropipette). The aliquot portions were then subject to analysis by chiral HPLC or if α–deutero-ibuprofenoyl-CoA was used, the samples were stored in the freezer (-20°C) where the samples were analysed at a later date with use of LCMS.

Example 2: 100μM 3-hydroxy-2-methyl-3-(4-nitrophenyl)propanoyl-CoA (**68**) with 100ng MCR in 1ml 10mM MOPS-CH_3CO_2Na buffer, pH 7.

From the aforementioned stock solution of MCR, 24μL was mixed with 913μL 10mM MOPS-CH_3CO_2Na buffer (made up of 91μL 100mM MOPS-CH_3CO_2Na buffer solution and 822μL H_2O). Again a separate solution of **68** was made using the same buffer as stated and calculated to have a concentration of 1.59mM from the extinction coefficient of the adenine

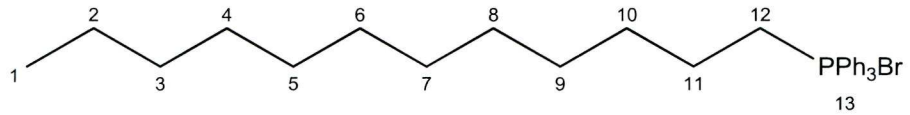
ring. The enzyme solution was placed in a 1ml quartz cuvette equipped with a magnetic stirrer and placed into a PerkinElmer Lambda 25 UV/VIS spectrometer. The temperature was set to 37°C and after 10 minutes the homogenised sample was subject to spectrophotometric analysis to allow for a 0 reading to be made. 63μL of the stock solution of the aromatic chromogen was then injected into the solution and a further measurement was made. Measurments were repeated over the course of 3 hours. The results of which can be seen in Fig 4.13

Example 3: 100μM (*S*)-ibuprofenoyl-CoA (**22a**) with 50 ng MCR in 1ml 10mM MOPS-CH_3CO_2Na buffer, pH 7.

As with example 1, the incubation was carried out at 37°C. At regular intervals 100μL was removed and added dropwise to 1M NaOH (500μL) This was allowed to stir at room temperature for 1 hour. After which time the mixture was acidifed with the dropwise addition of 1M HCl (550μL). The hydrolysed free acid was extracted into Et_2O (2 x 200μL) and subject to HPLC analysis.

5.12 Synthesis of Inhibitors

5.12.1 Dodecyltriphenylphosphonium bromide (**75**)[116]



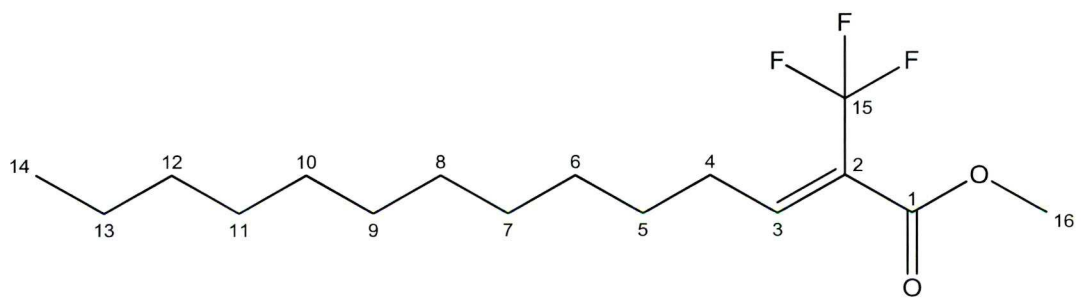
To 1-bromododecane (5.0ml, 20.66mmol) in anhydrous toluene (50ml) was added triphenylphosphine (4.41g, 20.66mmol). The solution was then heated under reflux for 48 hours after which time the solvent was removed under reduced pressure to yield the title compound as an off-white solid. Yield 8.74g, 98%.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 6.94 Hz, H-1), 1.19-1.38 (22H, m, H-2-12), 7.69-7.87 (15H, m, H-13).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.46 (C-1), 23.10 (C-2), 23.528 (C-3), 30.88-29.56 (C-4-11), 32.26 (C-12), 135.38-119.3161 (18C, C-13);

HRMS calcd for $C_{30}H_{40}P$ (M+) 431.2868; found 431.2881

5.12.2 Methyl 2-(trifluoromethyl)tetradec-2-enoate (**76**)[116]



1.6 M *n*-BuLi (2.2ml, 3.52mmol) was added dropwise to a cooled solution of compound **75** (1.38g, 3.2mmol) in THF (50 ml) at -78°C. The mixture was allowed to warm to room temperature over 2 hours and stirred for an additional 1 hour. The dark red mixture was again cooled to ca -78°C and trifluoromethyl pyruvate (0.5g, 3.2mmol) was added dropwise. The resulting yellow solution was monitored by TLC until conversion was complete. The mixture was then allowed to warm to room temperature, filtered through a short column of silica gel (30g) to remove the triphenylphosphine oxide and salts. The crude material was concentrated *in vacuo* and purified by silica gel flash chromatography using 1:4 Et_2O:hexane to yield both

geometric isomers (1:1) of the title compound as a pale yellow oil. Yield 0.23g, 23%. Rf = 069, 1:4 Et_2O:hexane;

(*E*)-isomer: ^1H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 7.0 Hz, H-14). 1.22-1.38 (16H, m, H-8-13), 1.41-1.52 (2H, m, H-5), 2.56-2.62 (2H, m, H-4), 3.82 (3H, s, H-16), 6.84 (1H, tq, $J_{3,4}$ = 7.4 Hz, H-3),

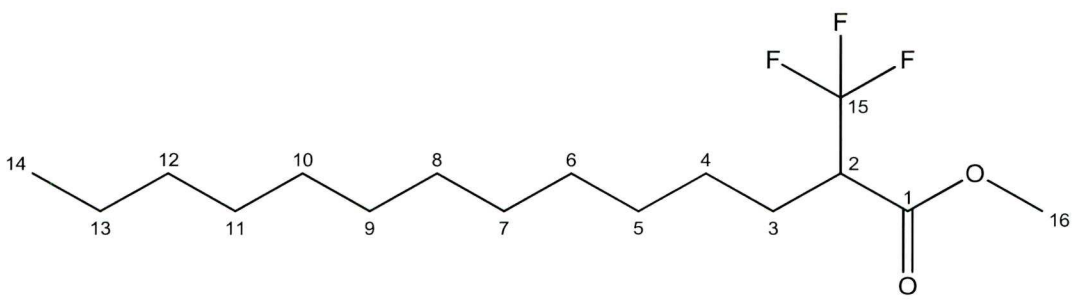
^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.5 (C-14). 23.0-32.3 (C-4-13), 52.3 (C-16), 128.9 (C-15), 151.1 (C-3), 169.1 (C-1),

(Z)-isomer: ^1H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 7.0 Hz, H-14). 1.22-1.38 (16H, m, H-8-13), 1.41-1.52 (2H, m, H-5), 2.42-2.48 (2H, m, H-4), 3.82 (3H, s, H-16), 7.21 (1H, tq, $J_{3,4}$ = 7.4 Hz, H-3),

^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.5 (C-14). 23.0-32.3 (C-4-13), 52.3 (C-16), 128.9 (C-15), 151.1 (C-3), 169.1 (C-1);

HRMS calcd for $C_{16}H_{31}F_3O_2N$ ($M+NH_4^+$) 326.2307; found 326.2301

5.12.3 Methyl 2-(trifluoromethyl)tetradecanoate (**78**)[116]



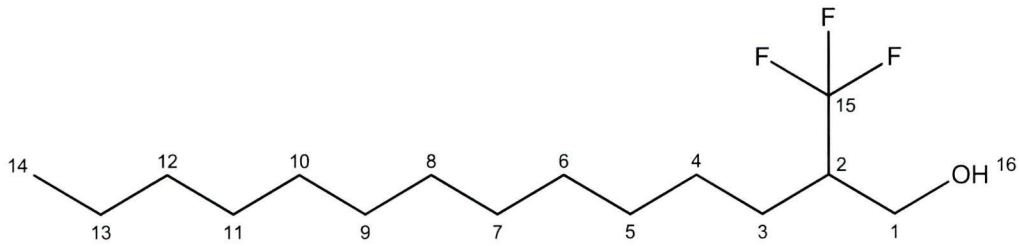
To a solution of compound **76** (0.4g, 1.5mmol) in distilled MeOH was added 1 micro spatula of palladium over activated carbon. The vessel was evacuated under reduced pressure and flushed 3 times with H_2 and stirred over night. It was then flushed with N_2 and opened to the atmosphere. The mixture was filtered through a pad of celite and washed with MeOH. The solvent was the concentrated *in vacuo* to yield the title compound. This gave to give the desired product as a yellow oily residue Yield 0.123g, 60%. Rf = 0.69, 1:4 Et_2O:hexane;

^1H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 7.0Hz, H-14), 1.22-1.38 (20H, m, H-4-13), 1.72-1.79 (1H, m, H-3), 1.83-1.91 (1H, m, H-3), 3.05-3.14 (1H, m, H-2), 3.77 (3H, s, H-16).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.41 (C-14). 23.03 (C-3), 26.50-32.28 (C-4-13), 50.57 (C-2), 52.83 (C-16), 126.50 (C-15), 168.54 (C-1),

HRMS calcd for $C_{16}H_{33}F_3O_2N$ ($M+NH_4^+$) 328.2463; found 328.2460

5.12.4 2-Trifluoromethyl)tetradecan-1-ol (**212**)



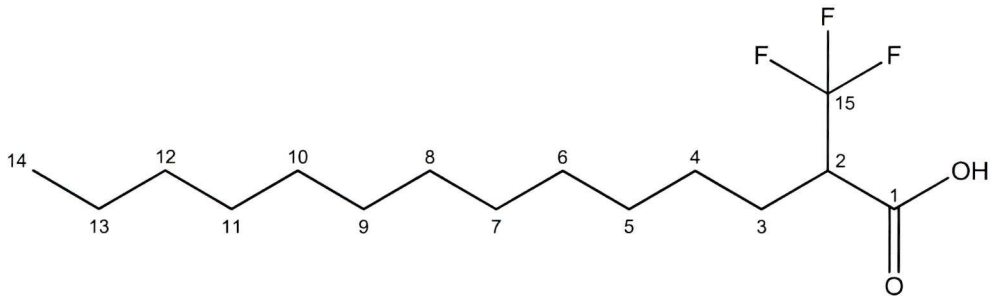
To compound **78** (0.1g, 0.322mmol) in MeOH (5 ml) at 0°C was added $LiAH_4$ (0.12g, 3.22mmol) in one portion. The mixture was allowed to warm to room temperature and stirred for 1 hr. H_2O (2ml) was added dropwise and MeOH was removed under reduced pressure. Et_2O (5ml) was then added, the aqueous layer was removed and the organic layer was washed with brine (10ml), followed by H_2O (10ml). The Et_2O layer was then dried over Na_2SO_4 and concentrated *in vacuo*. The crude oil was purified by silica gel flash chromatography, using 3:7 ethyl acetate/hexane to give the title compound as a colourless liquid. Yield 0.04g, 44%. Rf = 0.31, 1:4 Et_2O:hexane;

^1H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 6.84, H-14). 1.21-1.36 (16H, m H-5-13), 1.44-1.54 (2H, m, H-4), 1.59-1.72 (2H, m, H-3), 2.16-2.27 (1H, m, H-2), 3.81 (2H, d, J = 4.4 Hz H-1),

^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.46 (C-14), 23.06-32.30 (C-3-13), 46.01 (C-2), 60.37 (C-1), 129.78 (C-14).

(CI) m/z for $C_{15}H_2F_3O$ (M+): 282 (100.00)

5.12.5 .10 2-(Trifluoromethyl)tetradecanoic acid (**79**)



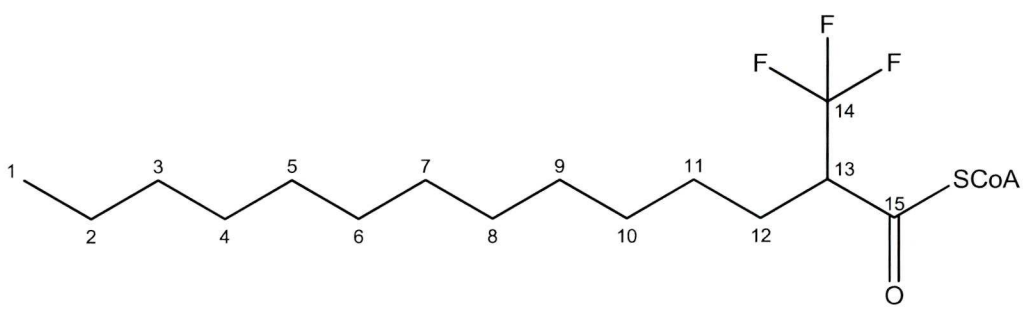
To a solution of compound **78** (0.1g, 0.32mmol) in wet DMF (1ml) was added lithium iodide (0.047g, 0.35mmol). This was heated to reflux for 12 hours. After which time the solution was diluted with water, acidified to pH 2 with the dropwise addition of 2M HCl and extracted with Et_2O (5ml). This was then dried over Na_2SO_4, concentrated *in vacuo* and purified using column chromatography using 2:5 Et_2O:hexane to yield the title compound as a pale yellow solid. Yield 0.058g, 61%. Rf = 0.18, 1:4 Et_2O:hexane;

^1H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 7.0 Hz, H-14), 1.22-1.36 (20H, m, H-4-13), 1.72-1.78 (1H, m, H-3), 1.83-1.92 (1H, m, H-3), 3.06-3.14 (1H, m, H-2);

δ ^{13}C (100MHz, $CDCl_3$) δ: 14.4 (C-14), 23.0-32.3 (C-3-13), 50.8 (C-2), 168.5 (C-1) ; υ_{max} /cm^{-1} 1756 (C=O), 3300-2950 (O-H acid).

HRMS calcd for $C_{15}H_{31}F_3O_2N$ ($M+NH_4^+$) 314.2307; found 314.2315

5.12.6.10 2-(Trifluoromethyl)tetradecanoyl-CoA (**48**)[116]

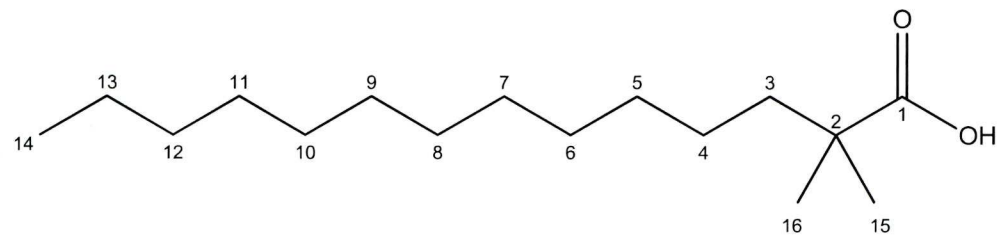


To a stirred solution of carbonyldiimidazole (28mg, 0.169mmol) desiccated over silica crystals overnight) in tetrahydrofuran (0.5ml) was added compound **79** (50mg, 16.87μmol). After 2 hours stirring at room temperature, a solution of CoA-SH, $(Li^+)_3$ (13.2mg,

0.169mmol) in water (0.5ml) was added. The resulting solution was allowed to stir at room temperature overnight. The product was then purified by preparative reverse phase HPLC. The crude material was dried by the removal of the organic solvent under reduced pressure, followed by removal of the aqueous solvent through lyophilisation to give the title compound as a white amorphous white solid (3.1mg, 21% yield- calculated from relative abundance of CoA-SH, $(Li^+)_3$;

HRMS calcd for $C_{36}H_{60}F_3N_7O_{17}P_3S$ $(M-H)^-$ 1044.2982; found 1044.2898

5.12.7,2-Dimethylpentadecanoic acid (**88**)

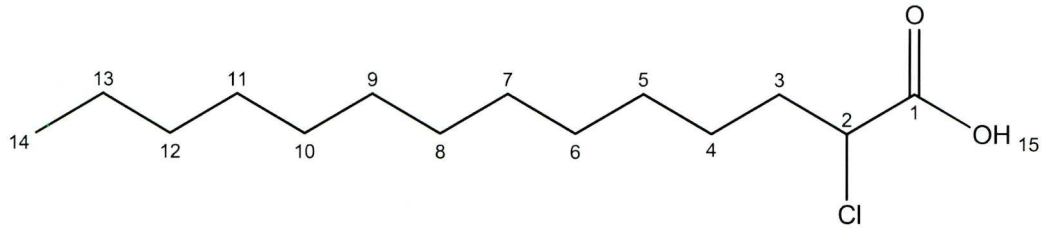


To a suspension of NaH (3.99g of 60% in oil, 100mmol) in anhydrous THF (83.3ml) and diisopropylamine (14.1ml, 100mmol) was added dropwise isobutyric acid (9.28ml, 100mmol). When gas evolution was complete, the mixture was heated and refluxed for 20 minutes. This was then cooled to 0°C and *n*-bromododecane (24ml, 100mmol) was added dropwise. The resulting solution was stirred for 60 minutes at 0°C and was allowed to warm to room temperature overnight. This was then re-cooled to 0°C and a minimum amount of H_2O was added. The solution was acidified pH 2 with the dropwise addition of conc HCl. Diethyl ether (50ml) was added and separated. The aqueous layer was further washed with ether (50ml) and the combined organic layers were washed with brine and dried over $MgSO_4$. Filtration and concentration afforded a white solid which was purified by silica gel flash chromatography using 1:9 EtOAc/hexane. Yield 14.8 g, 57.8%. Rf = 0.32, 1:9 EtOAc:hexane

^1H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* =6.85, H-14), 1.18-1.21 (6H, m, H-15-16), 1.21-1.30 (22H, m, H-4-13), 1.51-1.55 (2H, m, H-3).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.46 (C-14), 23.06 (C-13), 25.31 (C-15/16), 29.35-32.31 (C-4-12), 40.954 (C-3), 42.519 (C-2), 185.11 (C-1). υ_{max} /cm^{-1} 2915.84 (C-H), 1700.91 (C=O). (Cl) m/z for $C_{16}H_{35}O_2N$ $(M + NH_4^+)$: 274 (100.00)

5.12.8 2-Chlorotetradecanoic acid (**92**)



Myristic acid (5.0 g, 20.0 mmol) and thionyl chloride (6.39ml, 80.0mmol) were added to a reaction vessel and heated to reflux for 30 minutes. The reaction was monitored by TLC until the reaction had completed (95:5 dichloromethane/methanol). This was cooled to room temperature and to this was added *N*-chlorosuccinimide (5.34g, 40.0mmol), followed by conc HCl (2 drops) and an additional 4ml of thionyl chloride. This was then heated to 80°C for 1.25 hr. The solvent was then removed under reduced pressure and the remaining solid was washed with hexane (50ml) to remove the *N*-chlorosuccinimide. This was then re-washed with H_2O (2 x 50ml), dried over $MgSO_4$ and the remaining organic solvent was removed under reduced pressure.

The resulting α-chlorinated acid was added to a 1:1 mixture of THF and 2M HCl (4ml) and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting white solid was purified by flash column chromatography using 3:7 EtOAc/hexane. Yield 54%. Rf = 0.5, 3:7 EtOAc:hexane.

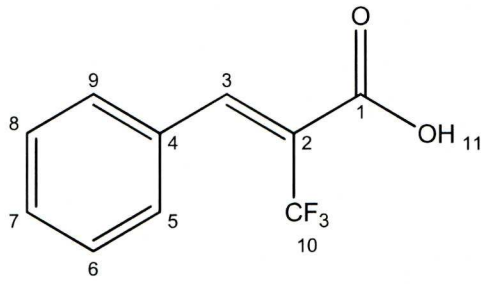
^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 13.16, H-14), 1.36-1.32 (18H, m, H-5-13), 1.51-1.39 (2H, m, H-4), 1.99-1.90 (1H, m, H-3), 2.08-2.00 (1H, m, H-3), 4.31 (1H, dd, J = 5.9 Hz, J = 6.94 Hz, H-2).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.46 (C-14), 23.06 (C-13), 26.28 (C-5), 30.005-29.205 (C-6-12), 32.29 (C-4), 35.18 (C-3), 57.52 (C-2), 175.31 (C-1).

(Cl) m/z for $C_{14}H_{31}{}^{37}ClO_2N$ ($M+NH_4^+$): 282 (100.00)

Elemental Analysis: Theoretal C: 63.98%, H: 10.35% Found C: 62.90%, H: 10.15%

5.12.9 (*E/Z*)-3-Phenyl-2-(trifluoromethyl)acrylic acid (**99**)



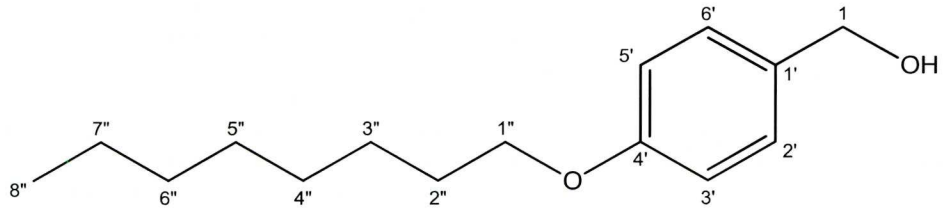
Triphenylphosphine (0.084g, 0.32mmol), palladium acetate (0.024g, 0.1mmol), caesium chloride (2.28g, 7.14mmol) and bromobenzene (1. 68ml, 10.71mmol) in 10ml dry DMF was added 2-(trifluoromethyl)-acrylic acid (0.5g, 3.57mmol) dropwise under a strict N_2 atmosphere. This was heated to 130°C over night. After which time the solution was acidified to pH 3-4 with the dropwise addition of 1M HCl and extracted with EtOAc (2 x 20ml). The crude product was purified by flash column chromatography using 4:6 EtOAc:hexane to yield the title compound as a brown solid. Yield 0.77 g, 67%. Rf = 0.21, 4:6 EtOAc:hexane;

(Z)-isomer ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.23-7.38 (5H, m, H-5,6,7,8,9), 7.79 (1H, s, 3H), ^{13}C NMR (100 MHz, $CDCl_3$) δ: 120.74, 124.31, 128.73-130.85, 132.67, 142.45, 167.69.

(E)-isomer ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.23--7.23 (5H, m, H-5,6,7,8,9), 7.71 (1H, s, H-3), ^{13}C NMR (100 MHz, $CDCl_3$) δ: 120.74, 124.31, 128.73-130.85, 132.67, 142.45, 167.69.

m/z for $C_{10}H_{10}F_3O_2N$ ($M + NH_4^+$): 234 (100.00)

5.12.10 (4-(Octyloxy)phenyl)methanol[177] (**104**)

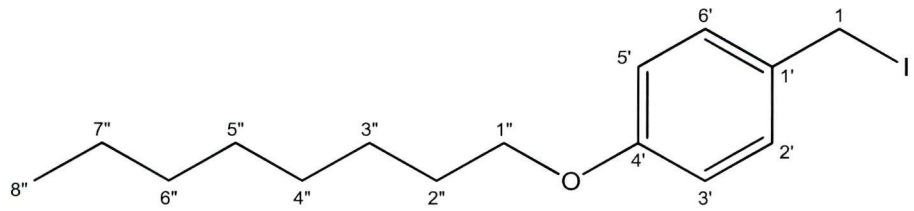


To 4-hydroxybenzyl alcohol (5g, 40.28 mmol) in DMF (40ml) was added potassium carbonate (5.57g, 40.30mmol) at room temperature. This was followed by the dropwise addition of 1-bromo-octane (7.65ml, 35.43 mmol). The solution was allowed to stir at that temperature for 20 minutes. This was then gradually brought to 60°C for 4 hours. The reaction was quenched with water (40ml), extracted with Et_2O (2 x 50mL), washed with brine

(50ml) and finally washed with water (50ml). The organic layer was then dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude solid was purified using column chromatography using 3:7 Et_2O: Hexane to yield a white solid. Yield 8.6g, 83%. Rf = 0.19, 3:7 Et_2O:hexane;

^1H (400MHz, $CDCl_3$) δ: 0.91 (3H, t, *J* = 7.45 Hz, H-8"), 1.27-1.58 (10H, m, H-2"-7") 3.20 (2H, m, H-1"), 3.79 (2H, s, H-1), 6.54 (2H, d, *J* = 8.83 Hz, H -3',5'), 6.76 (2H, d, *J* = 9.02 Hz, H-2',6');
^{13}C (100MHz, $CDCl_3$) δ: 14.53 (C-8"), 23.08-32.23 (C-2"-7"), 65.54 (C-1), 68.48 (C-1") 114.97 (C-3',5'), 129.06 (C-2',6'), 133.28 (C-1') 159.22 (C-4');
HRMS calcd for $C_{15}H_{25}O_2$ ($M+H^+$) 237.0891; found 237.0891.

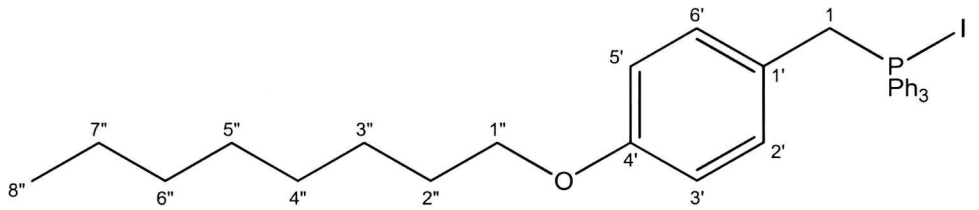
5.12.11 1-(Iodomethyl)-4-(octyloxy)benzene (**105**)



To a solution of triphenylphosphine (4.33g, 16.5mmol) in dry DCM (15ml) was added imidazole (1.12g, 16.5mmol) followed by iodine (4.19, 16.5mmol) under N_2. This was allowed to stir for 5 minutes. During which time the solution changed from a colourless solution, to a yellow, and finally to a bright orange solution with a white precipitate. To this was then added compound **104a** (3 g, 12.7 mmol) in one portion whilst still maintaining an inert atmosphere. This was allowed to stir at room temperature for 30 minutes. After which time the solid was filtered off and the organic layer was subsequently washed with saturated sodium thiosulfate (20ml), H_2O (20ml), and finally with brine (20ml). The organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The crude extract was mounted onto silica and purified using flash chromatography with an eluent system of 3:7 Et_2O:hexane to yield a white solid. Yield 4.35g, 98%. Rf = 0.67, 3:7 Et_2O:hexane
^1H (400MHz, $CDCl_3$) δ: 0.91 (3H, t, *J* = 7.45 Hz, H-8"), 1.27-1.58 (10H, m, H-2"-7") 3.20 (2H, m, H-1"), 3.79 (2H, s, H-1), 6.54 (2H, d, *J* = 8.83 Hz, H -3',5'), 6.76 (2H, d, *J* = 9.02 Hz, H-2',6');

^{13}C (100MHz, $CDCl_3$) δ: 7.26 (C-1), 14.53 (C-8") 23.08-32.23 (C-2"-7"), 68.48 (C-1"), 115.21 (C-3',5'), 129.81 (C-2','6'), 131.47 (C-1') 159.22 (C-2');

5.12.12 Iodo(4-(octyloxy)benzyl)triphenylphosphorane (**106**)



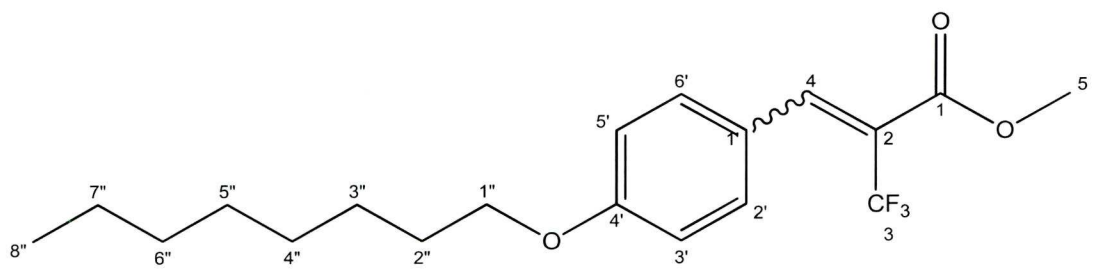
To compound **105a** (2.17 g, 6.26 mmol) and triphenylphosphine (2.46, 9.39 mmol) was added dry MeCN (10ml) under N_2. A white precipitate began to form almost immediately. This was then heated to reflux for 24 hours. After which time the reaction vessel was cooled to room temperature and the solvent was removed under reduced pressure. The gummy white solid was washed with hexane to remove and unreacted starting material. This was dried under vacuum overnight to yield a white solid. Yield 3.3g, 87%.

^1H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 6.88 Hz, H-8"), 1.22-1.50 (10H, m, H-3"-7"), 1.75 (2H, m, H-2") 3.20 (2H, m, H-1"), 3.79 (2H, s, H-1), 6.54 (2H, d, J = 8.83 Hz, H-3',5'), 6.76 (2H, d, J = 9.02 Hz, H-2',6');

^{13}C (100MHz, $CDCl_3$) δ: 14.53 (C-8") 21.87-32.19 (C-2"-7",1), 68.47 (C-1"), 115.30 (C-3',5'), 117.57-138.25 (C-1',2',6'), 159.22 (C-4');

HRMS calcd for $C_{33}H_{38}OP$ (M+) 481.2660; found 481.2645

5.12.13 (*E/Z*)-Methyl 3-(4-(octyloxy)phenyl)-2-(trifluoromethyl)acrylate (**107**)

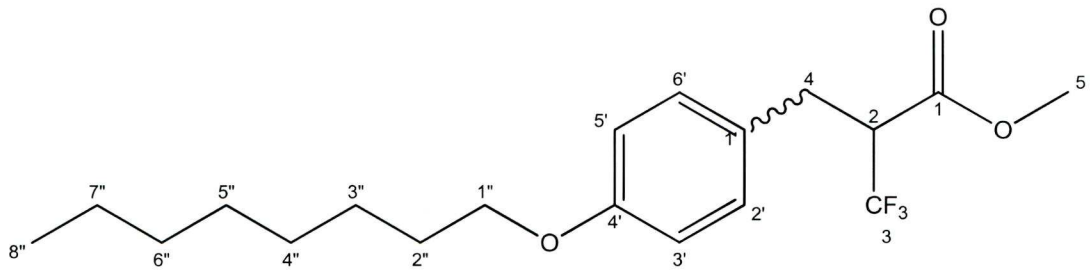


Iodo(4-(octyloxy)benzyl)triphenylphosphorane (**106**) (1.3g, 2.1mmol) was reacted according to the procedure described in Section 5.11.2 to yield the title compound **107** as a thick orange oil. The crude material was purified by silica gel flash chromatography using 1:9 Et_2O:hexane to yield the title compound as a pale yellow oil. Yield 0.26g, 34% Rf = 0.59, 2:8 Et_2O:hexane.

^{1}H (400MHz, $CDCl_3$) δ: 0.92 (3H, t, *J* = 6.79 Hz, H-8"), 1.27-1.55 (10H, m, H-3"-7") 1.83 (2H, m, H-2") 3.86 (3H, s, H-5), 4.01 (2H, t, *J* = 6.54 Hz, H-1"), 6.92 (2H, d, *J* = 8.82 Hz, H-3',5'), 7.36, (1H, s, H-4) 7.41 (2H, d, *J* = 8.83 Hz, H-2',6');

^{13}C (100MHz, $CDCl_3$) δ: 14.51 (C-8"), 23.08-32.23 (C-2"-7"), 52.89 (C-5), 68.40 (C-1") 114.94 (C-3',5'), 120.27 (C-3, q, J_{C-F} = 0.31 Hz), 132.04 (C-2',6') 134.24 (C-4), 140.82 (C-1'), 161.67 (C-4'), 164.721 (C-1); υ_{max} /cm^{-1} 2979.48 (C-H), 1749.09 (C=O), 1604 (C=C), HRMS calcd for $C_{19}H_{29}F_3O_3N$ ($M+NH_4^+$) 376.20995; found 376.21095.

5.12.14. Methyl 3,3,3-trifluoro-2-(4-(octyloxy)benzyl)propanoate (**108**)

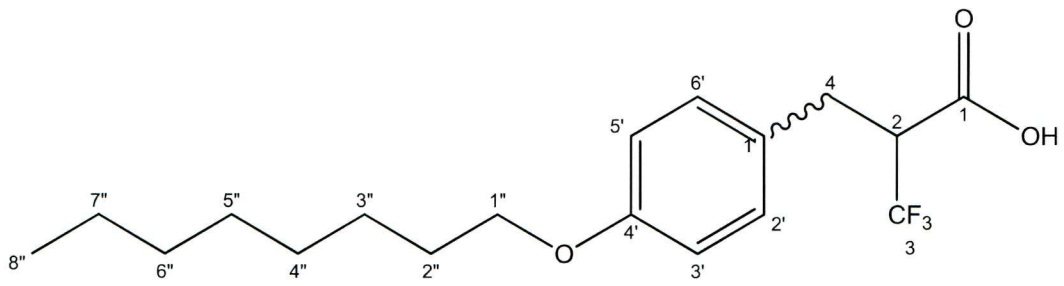


In a high pressure lab autoclave, a solution of methyl 3-(4-(octyloxy)phenyl)-2-(trifluoromethyl)acrylate (**107**) (0.150g, 0.42mmol) in distilled MeOH (5ml) was added 1 micro spatula of 5% platinum over activated carbon. The vessel was flushed 3 times with H_2, pressurised with H_2 to 800 psi and stirred overnight. After careful release of H_2, the mixture was filtered through a pad of celite and washed with MeOH. The solvent was the concentrated *in vacuo* and the crude material was purified by flash column chromatography with an eluent system of 2:8 Et_2O:hexane to yield the title compound as a white solid. Yield 0.135g, 89%. Rf = 0.59, 2:8 Et_2O:hexane.

^{1}H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 6.92 Hz, H-8"), 1.23-139 (10H, m, H-3"-7") 1.77 (2H, m, H-2") 3.08 (2H, dd *J* = 13.92, 4.58 Hz, H-4a,4b) 3.37 (1H, m, H-2) 3.65 (3H, s, H-5), 3.92 (2H, t, *J* = 6.60 Hz, H-1"), 6.82 (2H, d, *J* = 8.73Hz, H -3',5'), 7.08 (2H, d, *J* = 8.64 Hz, H-2',6');

^{13}C (100MHz, $CDCl_3$) δ: 14.51 (C-8"), 23.06-32.22 (C-2"-7"), 52.99 (C-5), 53.00 (C-2), 68.40 (C-1") 115.09 (C-3',5'), 128.26 (C-1'), 130.15 (C-2',6'), 158.69 (C-4'), 167.89 (C-1); HRMS calcd for $C_{19}H_{27}O_3F_3Na$ (M+Na) 383.1810; found 383.1792.

5.12.15 3,3,3-Trifluoro-2-(4-(octyloxy)benzyl)propanoic acid (**109**)



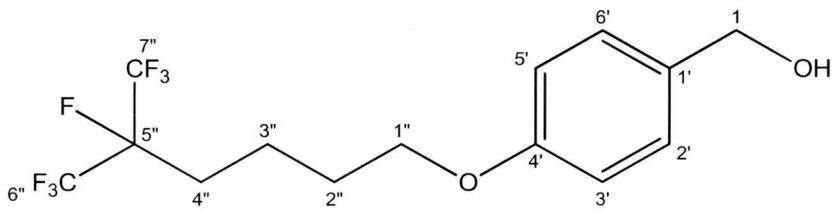
Methyl 3,3,3-trifluoro-2-(4-(octyloxy)benzyl)propanoate (**108**) (0.05g, 0.014mmol) was reacted according to the procedure described in Section 5.11.5 to yield the title compound as a white solid. Yield 0.033g, 70%. Rf = 0.32, 2:8 Et_2O:hexane

^1H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 6.91 Hz, H-8"), 1.23-139 (10H, m, H-3"-7") 1.77 (2H, m, H-2") 3.08 (2H, dd *J* = 13.85, 10.72, 4.65 Hz, H-4a,4b) 3.40 (1H, m, H-2), 3.92 (2H, t, *J* = 6.60 Hz, H-1"), 6.82 (2H, d, *J* = 8.73Hz, H -3',5'), 7.08 (2H, d, *J* = 8.64 hz, H-2',6');

^{13}C (100MHz, $CDCl_3$) δ: 14.51 (C-8"), 23.00-32.19 (C-2"-7"), 55.25 (C-2), 68.40 (C-1") 115.09 (C-3',5'), 128.26 (C-1'), 130.15 (C-2'6'), 158.70 (C-4'), 173.52 (C-1);

HRMS calcd for $C_{18}H_{25}O_3F_3Na$ (M+Na) 369.1653; found 369.1639.

5.12.16 (4-(6,7,7,7-Tetrafluoro-6-(trifluoromethyl)heptyl)phenyl)methanol (**104a**)



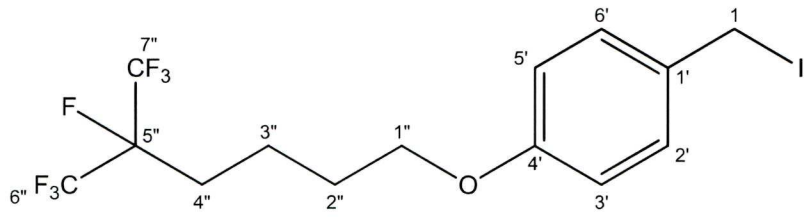
To 4-hydroxy benzyl alcohol (0.69g, 6.1mmol) was reacted according to the procedure described in Section 5.11.10, using DMF (10ml), potassium carbonate (1.53g, 11.07mmol) and 1,1,1,2-tetrafluoro-2-trifluoromethyliodohexane (2g, 5.68mmol). Again the crude material was purified using flash column chromatography using 3:7 Et_2O:hexane to yield a white solid. Yield 1.83g, 86%

^1H (400MHz, $CDCl_3$) δ: 1.81 (4H, m, H3"-4"), 2.15 (2H, m, H-2"), 3.99 (2H, t, *J* = 5.80 Hz, H-1"), 4.59 (2H, s, H-1) 6.87 (2H, d, *J* = 8.58 Hz, H-2',6') 7.28 (2H, d, *J* = 8.52 Hz, H-3',5')

^{13}C (100MHz, $CDCl_3$) δ: 18.94 (C-4"), 29.10-29.59 (C-2",3"), 65.37 (C-1), 67.54 (C-1") 92.50 (C-6"-7"), 114.91 (C-3',5'), 121.20 (C-5"), 129.08 (C-2',6'), 133.71 (C-1') 158.82 (C-4').

HRMS calcd for $C_{14}H_{15}O_2F_7Na$ (M+Na) 371.0858; found 371.0858

5.12.17 1-(Iodomethyl)-4-(5,6,6,6-tetrafluoro-5-(trifluoromethyl)heptyl)benzene (**105a**)

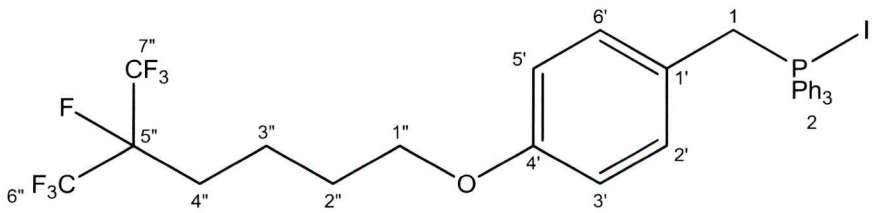


Compound **104a** was reacted according to the procedure described in Section 5.11.11. The crude extract was mounted onto silica and purified using flash chromatography with an eluent system of 3:7 Et_2O:hexane to yield a white solid. Yield 1.18g, 90%.

^1H (400MHz, $CDCl_3$) δ: 1.81 (4H, m, H3"-4"), 2.15 (2H, m, H-2"), 3.99 (2H, t, J = 5.80 Hz, H-1"), 4.47 (2H, s, H-1) 6.87 (2H, d, J = 8.71 Hz, H-2',6') 7.28 (2H, d, J = 8.52 Hz, H-3',5')

^{13}C (100MHz, $CDCl_3$) δ: 14.52 (C-4"), 23.07 (C-1), 26.46-32.23 (C-2"-3") 68.39 (C-1"), 115.08 (C-3',5'), 128.25 (C-1'), 128.90 (C-5"), 130.16 (C-2',6'), 132.41 (C-6"-7"), 160.71 (C-1') 158.68 (C-4').

5.12.18 Iodotriphenyl(4-(6,7,7,7-tetrafluoro-6-trifluoromethyl)heptyl)benzyl)phosphorane (**106a**)



Compound **105a** (1g, 2.18mmol) was reacted according to the procedure described in Section 5.11.12. The gummy white solid was washed with hexane to remove and unreacted starting material. This was dried under vacuum overnight to yield a white solid. Yield 1.41g, 90%

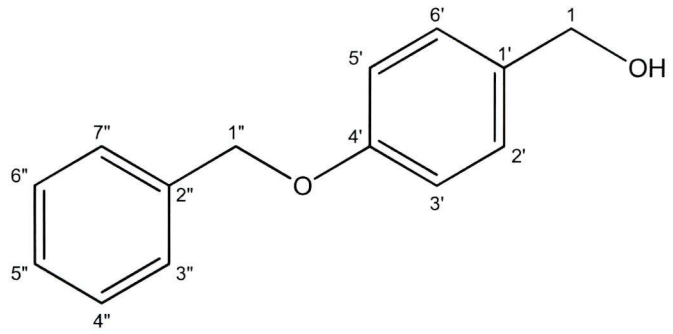
^1H (400MHz, $CDCl_3$) δ: 1.74 (6H, m, H3"-4"), 2.15 (2H, m, H-2"), 3.90 (2H, t, J = 5.60 Hz, H-1"), 5.18 (2H, d, J = 13.57 Hz, H-1), 6.64-7.79 (19H, m, H-2',3',5',6',2).

^{13}C (100MHz, $CDCl_3$) δ: 18.92 (C-3"), 29.04-31.07 (C-2"-4",1), 67.53 (C-1") 115,19-118.72 (C-3'5',5"),), 128.97 (C1')133.16 (C-2',6'), 134.79 (C-2), 135.50 (C-2), 159.36 (C-4')

υ_{max} /cm^{-1} 1232.29 (C-O), 2983.34 (C-H)

HRMS calcd for $C_{32}H_{29}OF_7P$ (M+) 593.1844; found 593.1855.

5.12.19 4-(Benzyloxy)phenylmethanol[178] (**118**)



To a mixture of 4-hydroxy benzyl alcohol (4g, 32.22mmol) and potassium carbonate (4.45g, 32.22mmol) in dry acetone (100ml) was added benzyl bromide (5.51g, 3.83ml, 32.22mmol) in portions. This was allowed to reflux for 4 hours. After completion (by TLC) the solution was cooled to room temperature and filtered. The collected solid was washed with ether. The combined ether and acetone solutions were evaporated to dryness and the re-dissolved in ether (100ml). This was then washed with 1M HCl (50ml), followed by H_2O (2 x 100ml). The ether layer was the dried over $MgSO_4$, filtered and the solvent was then removed under reduced pressure. The crude product was then purified using flash column chromatography using an eluent system of 1:9 Et_2O:hexane to yield the titled compound as a white solid. Yield 6.28g, 91%. Rf = 0.30, 1:9 Et_2O:hexane.

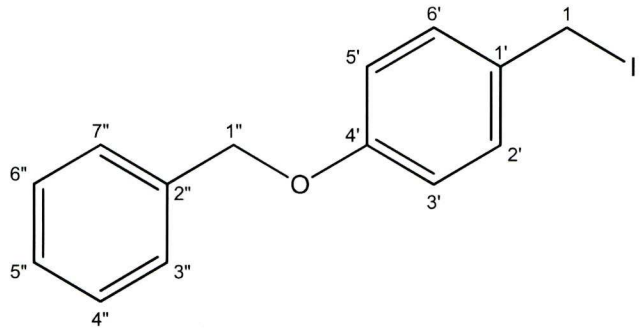
^1H (400MHz, $CDCl_3$) δ: 4.62 (2H, s, H-1), 5.07 (2H, s, H-1"), 6.98 (2H, d, J = 8.66 Hz, H-3',5'), 7.26-7.44 (7H, H-3",4",5",6",7",2',6').

^{13}C (100MHz, $CDCl_3$) δ: 65.48 (C-1), 70.44 (C-1"), 115.35 (C-3'.5'), 127.86-129.09 (C-3",4",5",6",7",2',6'). 133.78 (C-1'), 137.34 (C-2"), 158.82 (C-4').

υ_{max} /cm^{-1} 3365.17 (OH), 3027.69 (C-H), 1504.2 (C=C), 1236 (C-O).

HRMS calcd for $C_{14}H_{14}O_2Na$ (M+Na) 237.0891 found 237.0891

5.12.20 1-(Benzyloxy)-4-(iodomethyl)benzene (**119**)



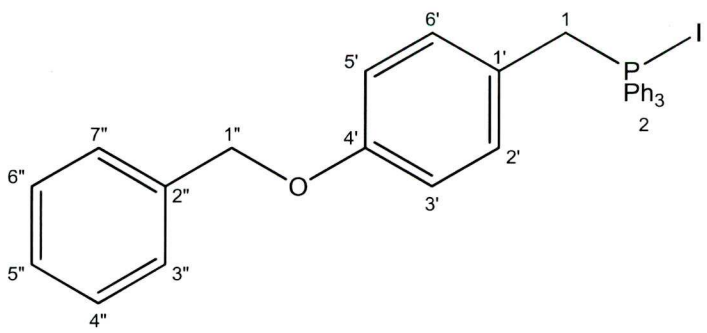
Compound **119** was prepared in a similar manner to the other aryl ether derivatives (**105/105a**) using triphenylphosphine (3.18g, 12.13mmol), imidazole (0.83g, 12.13mmol), iodine (1.54g, 12.13mmol) and 4-(benzyloxy)phenyl)methanol (**118**) (2g, 9.33mmol). The crude extract was mounted onto silica and purified using flash chromatography with an eluent system of 3:7 Et_2O:hexane to yield a white solid. Yield 2.72g, 90%. Rf = 0.72, 1:9 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 4.47 (2H, s, H-1), 5.04 (2H, s, H-1"), 6.90 (2H, d, J = 8.73 Hz, H-3',5'), 7.299-7.43 (7H, H-3",4",5",6",7",2',6').

^{13}C (100MHz, $CDCl_3$) δ: 6.98 (C-1), 70.46 (C-1"), 115.57 (C-3'-5'), 127.89-130.49 (C-3",4",5",6",7",2',6'), 132.02 (C-1'), 137.13 (C-2"), 158.81 (C-4').

υ_{max} /cm^{-1} 3027.69 (C-H), 1240.00 (C-O), 1506.13 (C=C).

5.12.21 (4-(Benzyloxy)benzyl)iodotriphenylphosphorane (**120**)



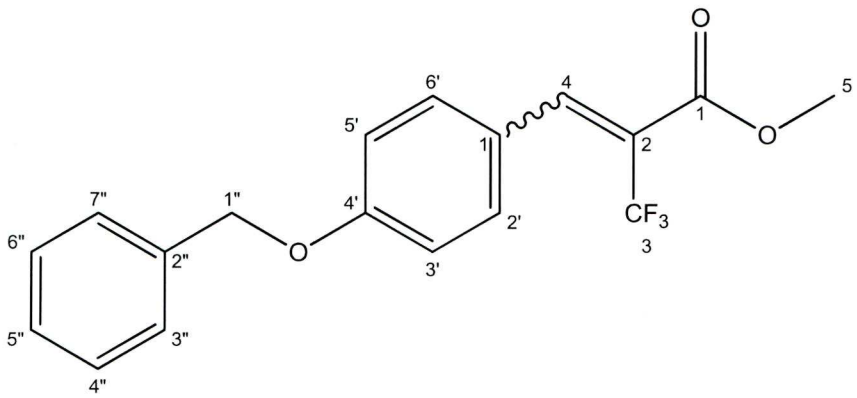
1-(Benzyloxy)-4-(iodomethyl)benzene (**119**) (2g, 6.17mmol) was reacted in accordance to the procedure carried out in Section 5.11.12 using 1.62g, 6.17mmol, triphenylphosphine. The gummy white solid was washed with hexane to remove and unreacted starting material. This was dried under vacuum overnight to yield a white solid. Yield 3.25g, 90%.

^{1}H (400MHz, $CDCl_3$) δ: 4.98 (2H, s, H-1"), 5.19 (2H, d, *J* = 13.57, H-1), 6.75 (2H, d, *J* = 8.41 Hz, H-3',5'), 6.99-7.02 (2H, dd, *J* = 8.8 Hz, 2.49, H-2'-6') 7.26-7.80 (20H, H-3",4",5",6",7",2).

^{13}C (100MHz, $CDCl_3$) δ: 6.98 (C-1), 70.46 (C-1"), 115.57 (C-3'-5'), 127.89-130.49 (C-3",4",5",6",7",2',6'), 132.02 (C-1'), 137.13 (C-2"), 158.81 (C-4').

HRMS calcd for $C_{32}H_{28}OP$ (M+) 459.1878; found 459.1897

5.12.22 (*E/Z*)-Methyl 3-(4-(benzyloxy)phenyl)-2-(trifluoromethyl)acrylate (**121**)



Compound **120** (1g, 1.7mmol) was reacted in accordance to the procedure described in Section 5.11.2, using 2.5M *n*-BuLi (0.75ml,1.88mmol) and trifluoromethyl pyruvate (0.174g, 0.17ml, 1.7mmol). The crude material was concentrated *in vacuo* and purified by flash column chromatography using an eluent system of 2:8 Et_2O:hexane to yield the title compound as a pale yellow oil. Yield 0.26g, 45%. Rf = 0.3, 1:9 Et_2O: Hexane

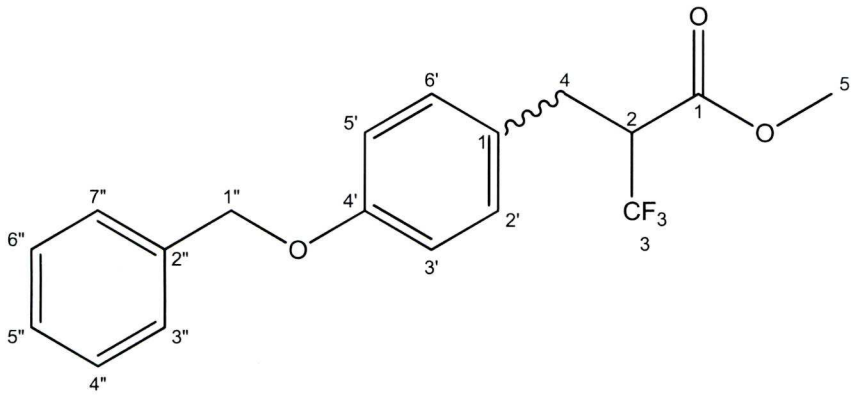
^{1}H (400MHz, $CDCl_3$) δ: 3.71 (2H, s, H-5), 4.99 (2H, s, H-1"), 6.87 (2H, d, *J* = 8.93 Hz, H-3',5'), 7.23-7.33 (8H, H-3",4",5",6",7",2',6',4).

^{13}C (100MHz, $CDCl_3$) δ: 52.97 (C-5), 70.50 (C-1"), 115.36 (C-3'-5'), 120.62-121.55 (C-3), 127.35-132.78 (C-3",4",5",6",7",1',2',6'), 13.69 (C-1"), 140.73 (C-4), 161.23 (C-4'), 164.67 (C-1).

υ_{max} /cm^{-1} 2915.84 (C-H), 1702.84 (C=O), 1606.41 (C=C), 1245.79 (C-O)

HRMS calc for C18H15O3F3Na (M+Na) 359.1009; found: 359.0877

5.12.23 Methyl 2-(4-(benzyloxy)benzyl)-3,3,3-trifluoropropanoate (**122**)



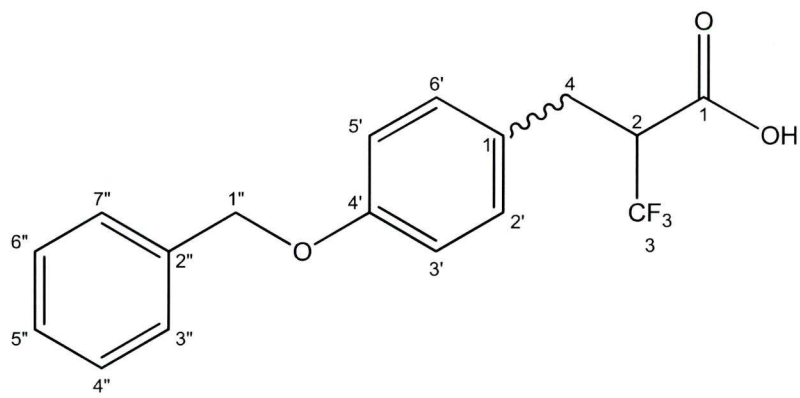
Compound **122** was synthesised in an analogous method to compounds **108/108a** to yield a pale yellow oil. Yield = 0.19g, 95%. Rf = 0.35, 1:9 Et_2O: hexane.

^1H (400MHz, $CDCl_3$) δ: 3.09 (2H, m, H-4), 3.36 (1H, m, H-2), 3.63 (3H, s, H-5), 5.02 (2H, s, H-1"), 6.90 (2H, d, *J* = 8.64 Hz, H-3',5'), 7.12 (2H, d, *J* = 8.60 Hz H-2',6') 7.30-7.43 (5H, H-2",3",4",5",6",7").

^{13}C (100MHz, $CDCl_3$) δ: 31.78 (C-4) 52.95 (C-2) 53.05 (C-5), 70.40 (C-1"), 115.48 (C-3',5'), 127.94-130.28 (C-3",4",5",6",7",1',2',6',3), 137.30 (C-2"), 158.34 (C-4'), 167.86 (C-1).

HRMS calcd for $C_{18}H_{17}F_3O_3Na$ (M+Na) 361.1027; found 361.0998

5.12.24 2-(4-(Benzyloxy)benzyl)-3,3,3-trifluoropropanoic acid (**123**)



Methyl 2-(4-(benzyloxy)benzyl)-3,3,3-trifluoropropanoate (**122**) (0.1g, 0.29mmol) was reacted according to the procedure described in Section 5.11.5. Purification by flash column

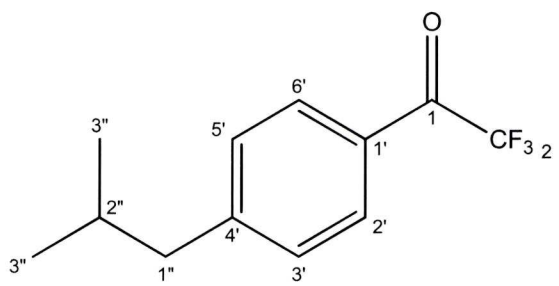
chromatography using 2:8 Et_2O:hexane afforded the title compound as a white solid. Yield 0.06g, 63% Rf = 0.37, 8:2 Et_2O: Hexane;

^1H (400MHz, $CDCl_3$) δ: 3.09 (2H, m, H-4), 3.37 (1H, m, H-2), 5.01 (2H, s, H-1"), 6.90 (2H, d, *J* = 8.64 Hz, H-3',5'), 7.12, (2H, d, *J* = 8.60 Hz H-2',6') 7.30-7.43 (5H, H-2",3",4",5",6",7").

^{13}C (100MHz, $CDCl_3$) δ: 18.62 (C-4) 52.81 (C-2) 70.41 (C-1"), 115.48 (C-3',5'), 127.90-129.00 (C-3",4",5",6",7",1',2',6'), 137.27 (C-2"), 158.37 (C-4'), 171.10 (C-1).

HRMS calcd for $C_{17}H_{15}F_3O_3Na$ (M+Na) 347.0870; found 347.0885

5.12.25 2,2,2-Trifluoro-1-(4-isobutylphenyl)ethanone (**135**)



To a stirred solution of $AlCl_3$ (10.9g, 81.7mmol) in DCM (30ml) under N_2 was added a mixture of trifluoroacetic anhydride (7.8g, 5.17ml, 37.2mmol) and isobutyl benzene (6.87g, 5.86ml, 37.2mmol) dropwise over the course of an hour at 0°C. The reaction was allowed to stir at that temperature for an additional 60 minutes. The reaction mixture was quenched by carefully pouring onto ice and acidified to pH 1 with conc HCl. The organic compounds were extracted with DCM (2 x 100ml) and washed with 2*N* NaOH (2 x 50ml) followed by brine (100ml). The combined organic layers were then dried over Na_2SO_4, concentrated *in vacuo* and chromatographed on silica gel using an eluent system of 1:9 Et_2O:hexane to afford the title compound as a pale yellow oil. Yield = 6.3g, 74%. Rf = 0.52, 1:9 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 0.93 (6H, d, *J* = 6.62 Hz, H-3"), 1.89-1.96 (1H, m, H-2"), 2.58 (2H, d, *J* = 7.18 Hz, H-1"), 7.32 (2H, d, *J* = 10.28 Hz, H-2',6'), 7.99 (2H, d, *J* = 7.64 Hz, H-3',5');

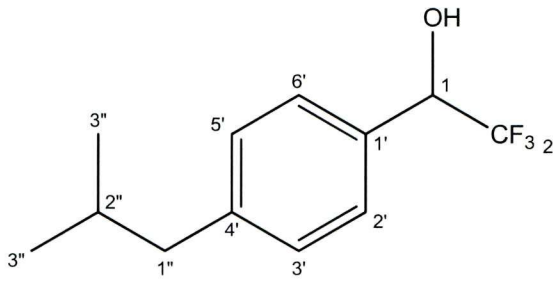
^{13}C (100MHz, $CDCl_3$) δ: 22.70 (C-3"), 30.48 (C-2"), 45.95 (C-1"), 117.18 (q, *J* = 2.90, C-2), 128.08 (C-1'), 130.24 (C-3',5'), 130.55 (C-2',6'), 151.11 (C-4'), 180.38 (q, *J* = 0.35 Hz, C-1)

;

υ_{max} /cm^{-1} 1712.48 (C=O), 2958.27 (C-H);

HRMS calcd for $C_{12}H_{17}F_3ON$ ($M+NH_4^+$) 248.12622; found 248.12606

5.12.26 2,2,2-Trifluoro-1-(4-isobutylphenyl)ethanol (**136**)



To compound **135** (2.69g, 11.7mmol) in ethanol (20ml) was added sodium borohydride (0.44g, 23.4mmol) at 0°C portionwise. This was allowed to react for 30 minutes. After which time the solvent was removed. Et_2O (50ml) was then added and acidified to pH 3 with the addition of 1M HCl (5ml). The organic layer was separated and washed with brine (50ml) followed by H_2O (50ml). It was then dried over Na_2SO_4 and concentrated *in vacuo*. Purification by flash column chromatography yielded the title compound as a yellow oil. Yield 2.44g, 90%. Rf = 0.26, 1:9 Et_2O:hexane.

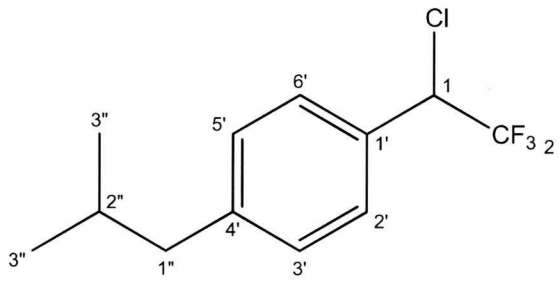
^{1}H (400MHz, $CDCl_3$) δ: 0.90 (6H, d, J = 6.61 Hz, H-3"), 1.73-1.83 (1H, sept, J = 6.79 H-2"), 2.40 (2H, d, J = 7.21 Hz, H-1"), 4.86 (1H, q, J = 6.90 Hz, H-1), 7.09 (2H, d, J = 8.11 Hz, H-2',6'), 7.27 (2H, d, J = 8.01 Hz, H-3',5')

^{13}C (100MHz, $CDCl_3$) δ: 22.73 (C-3"), 30.58 (C-2") 45.54 (C-1"), 73.20 (q, J = 31.92 Hz, C-1), 124.90 (q, J = 281.93 Hz, C-2) 127.63 (C-3',5'), 129.79 (C-2',6'), 131.72 (C-1'), 143.75 (C-4')

υ_{max} /cm^{-1} 2965.98 (C-H), 3397.96 (OH).

HRMS calcd for $C_{12}H_{19}F_3ON$ ($M+NH_4^+$) 250.14187; found 250.14238

5.12.27. 1-(1-Chloro-2,2,2-trifluoroethyl)-4-isobutylbenzene (**137**)



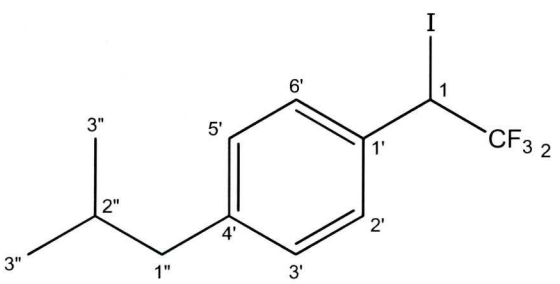
To compound **136** (0.4g, 1.72mmol) in 10ml DCM was added thionyl chloride (5ml, 0.0258moles). This was allowed to reflux for 4 hours. After which time the solvent and excess thionyl chloride was removed under reduced pressure. The remaining oil was extracted into diethyl ether (20ml) and washed with H_2O (2 x 20ml). The combined organic extracts dried over Na_2SO_4 and removed under reduced pressure. The compound was then purified by flash column chromatography to yield the title compound as a yellow oil. Yield 0.34g, 80% Rf = 0.73, 1:9 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) 0.90 (6H, d, J = 6.53 Hz, H-1), 1.76-1.89 (1H, sept, J = 6.72 H-2), 2.49 (2H, d, J = 7.28 Hz, H-1"), 5.08 (1H, q, J = 6.90 Hz, H-1), 7.17 (2H, d, J = 8.21 Hz, H-6,8), 7.39 (2H, d, J = 8.21 Hz, H-5,9).

^{13}C (100MHz, $CDCl_3$) δ: 22.71 (C-3"), 30.54 (C-2") 45.52 (C-1"), 59.10 (q, J = 34.13 Hz, C-1), 124.00 (q, J = 279.11 Hz, C-2) 128.86 (C-3',5'), 129.0 (C-1',2',6'), 144.41 (C-4') υ_{max} /cm^{-1} 2965.98 (C-H).

HRMS calcd for $C_{12}H_{14}{}^{35}ClF_3$(M+) 250.07361; found 250.07389

5.12.28 1-Isobutyl-4-(2,2,2-trifluoro-1-iodoethyl)benzene (**138**)



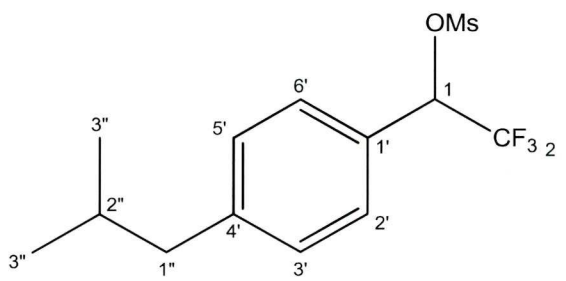
To a solution of triphenylphosphine (0.47g, 2.32mmol), in dry DCM (15ml) was added imidazole (0.12g, 2.32mmol) followed by iodine (0.45g, 2.32mmol) under N_2. This was allowed to stir for 5 minutes. During which time the solution changed from a colourless

solution, to a yellow, and finally to a bright orange solution with a white precipitate. To this was then added 2,2,2-trifluoro-1-(4-isobutylphenyl)ethanol (**136)** (0.415g, 1.79mmol) in one portion whilst still maintaining an inert atmosphere. This was allowed to stir at room temperature for 30 minutes. The temperature was then increased to 60°C for 2 hours. After which time the temperature was reduced to 25°C and the solid was filtered off. The organic layer was subsequently washed with saturated sodium thiosulfate (3 x 20ml), H_2O (2 x 20ml), and finally with brine (20ml). The organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The crude extract was mounted onto silica and purified by column chromatography (100% hexane) to yield the title compound as a yellow oil. Yield 0.59g, 74%, Rf = 0.78, 100% hexane.

^{1}H (400MHz, $CDCl_3$) δ: 0.83 (6H, d, *J* = 6.61 Hz, H-3"), 1.76-1.89 (1H, m, H-2"), 2.42 (2H, d, *J* = 7.19 Hz, H-1"), 4.91 (1H, q, *J* = 6.71 Hz, H-2), 7.16 (2H, d, *J* = 8.06 Hz, H-2',6'), 7.30 (2H, d, *J* = 7.95 Hz, H-3',5')

^{13}C (100MHz, $CDCl_3$) 22.76 (C-3"), 30.59 (C-8) 45.23 (C-2"), 73.20 (q, *J* = 31.93, C-1), 127.62 (C-3',5'), 129.80 (C-2',6'), 130.27 (C-1') 143.76 (C-4')

5.12.29 2,2,2-Trifluoro-1-(4-isobutylphenyl)ethyl methanesulfonate (**139**)

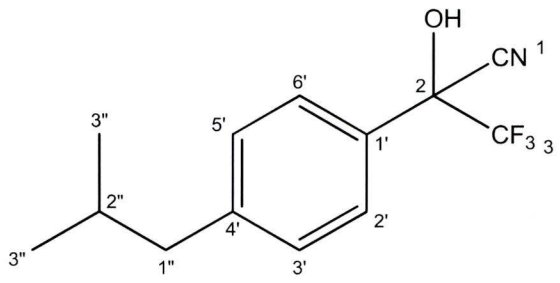


To compound **136** (0.7g, 3.0mmol) in DCM (3ml) was added triethylamine (0.839ml, 6 mmol) under N_2. After 30 minutes of stirring at this temperature mesyl chloride (0.23ml, 3mmol) was added dropwise and the reaction was allowed to stir at that temperature for a further hour. The reaction was quenched with H_2O (5ml), and separated. The organic layer was then washed with brine (5ml) followed by H_2O (2 x 10ml) and dried over Na_2SO_4. The solvent was then removed under reduced pressure and the crude colourless oil was then purified by column chromatography using 1:9 Et_2O:hexane to yield the title compound as a colourless oil. Yield 0.82g, 88%. Rf = 0.3, 1:9 Et_2O:hexane.

^{1}H (400MHz, $CDCl_3$) δ: 0.90 (6H, d, *J* = 6.53 Hz, H-3"), 1.83-1.93 (1H, sept, *J* = 6.72 H-2"), 2.50 (2H, d, *J* = 7.16 Hz, H-1"), 2.93 (3H, s, OMs) 5.75 (1H, q, *J* = 6.38 Hz, H-1), 7.23 (2H, d, *J* = 8.15 Hz, H-2',6'), 7.41 (2H, d, *J* = 8.00 Hz, H-3',5')

^{13}C (100MHz, $CDCl_3$) δ: 22.72 (C-3"), 30.49 (C-2"), 39.83 (OMs) 45.54 (C-1"), 78.70 (q, *J* = 34.45 Hz, C-1), 123.00 (q, *J* = 280.64 Hz, C-2) 126.97 (C-1') 128.36 (C-3',5'), 130.20 (C-2',6'), 145.22 (C-4')

5.12.30 3,3,3-Trifluoro-2-hydroxy-2-(4-isobutylphenyl)propanenitrile (**142**)

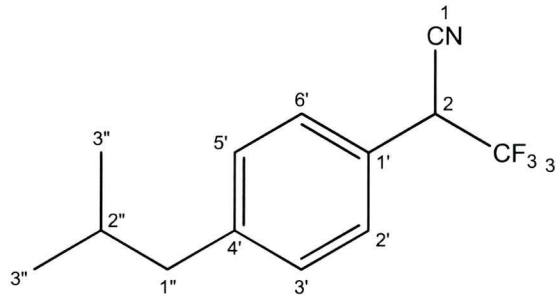


To, compound **135** (1g, 4.3mmol) in distilled H_2O (10ml) was added sodium bisulfite (1.48g, 8.6mmol) at room temperature. This was allowed to stir for 1 hour. After which time a white precipitate was formed and sodium cyanide (0.42g, 8.6mmol) was then added. The precipitate then disappeared over the course of an hour and to this was added Et_2O (10ml). The aqueous layer was separated and washed with ether three more times. The combined organic layers were dried over Na_2SO_4 and removed under reduced pressure to yield the title compound as a yellow oil which was used without further purification. Yield 0.55g, 50%

^{1}H (400MHz, $CDCl_3$) δ: 0.95 (6H, d, *J* = 6.64 Hz, H-3"), 1.86-1.96 (1H, sept, *J* = 6.77 H-2"), 2.55 (2H, d, *J* = 7.74 Hz, H-1"), 4.13 (1H, s, OH), 7.29 (2H, d, *J* = 8.01 Hz, H-2',6'), 7.61 (2H, d, *J* = 8.10 Hz, H-3',5')

^{13}C (100MHz, $CDCl_3$) δ: 22.70 (C-3"), 30.51 (C-2"), 45.42 (C-1"), 74.40 (q, *J* = 33.75 Hz, C-2), 116.09 (C-1) 122.00 (q, *J* = 286.23 Hz, C-3) 126.76 (C-3',5') 128.31 (C-1') 130.20 (C-2',6'), 145.57 (C-4').

5.12.31 3,3,3-Trifluoro-2-(4-isobutylphenyl)propanenitrile (**140**)

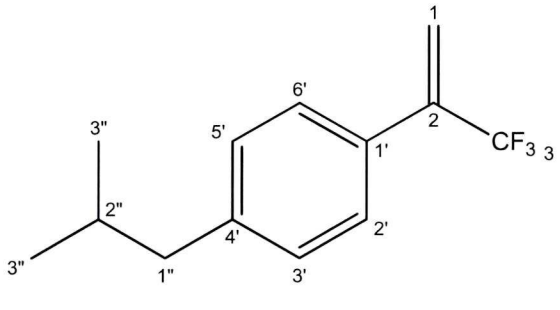


To compound **142** (0.1g, 3.88mmol) in toluene (1ml) was added phosphorus pentasulfide (0.17g, 3.88mmol) and allowed to reflux for 4 hours. The solid was filtered off and washed with toluene. The filtrate was diluted with Et_2O (2ml) and washed with 1 *N* sodium hydrogen carbonate solution (2ml) followed by water (2ml). The organic layer was dried over Na_2SO_4 and evaporated to dryness to yield the desired compound as a yellow oil. No further purification was necessary. Yield 0.54g, 58%.

^1H (400MHz, $CDCl_3$) δ: 0.91 (6H, d, *J* = 6.62 Hz, H-3"), 1.83-1.93 (1H, sept, *J* = 6.79 H-2"), 2.52 (2H, d, *J* = 7.24 Hz, H-1"), 4.44 (1H, q, *J* = 7.59 Hz, H-2), 7.23 (2H, d, *J* = 8.16 Hz, H2',6'), 7.36 (2H, d, *J* = 8.16 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 22.69 (C-3"), 30.52 (C-2"), 43.30 (C-1), 45.39 (C-1"), 116.09 (C-2), 129.27 (C-3',5') 130.51 (C-2',6').

5.12.32 1-Isobutyl-4-(3,3,3-trifluoroprop-1-en-2-yl)benzene (**146**)



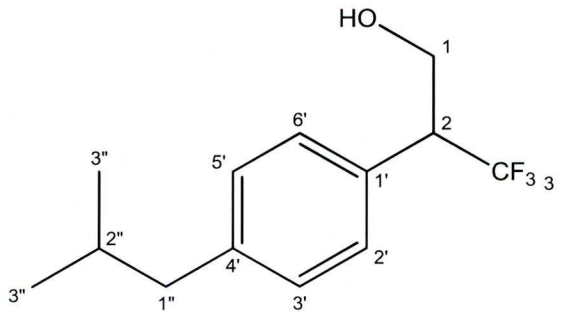
Triphenylphosphine methyl iodide (1.76g, 4.35mmol) in THF (35ml) at -78°C under N_2 was added 2.5M *n*-BuLi (1.911ml, 4.78mmol). This was allowed to stir at that temperature for 1 hour and then allowed to warm to room temperature over the course of 2 hours. The temperature was then reduced to -78°C and compound **135** was added dropwise (1g,

4.35mmol). This was allowed to stir at that temperature for 1 hour. After which time the mixture was then warmed to room temperature and filtered through a short column of silica gel (30g) to remove triphenylphosphine oxide and salts. The crude product was concentrated *in vacuo* and chromatographed on silica gel using an eluent system of 1:20 Et_2O:hexane to yield the title compound as a pale yellow. Yield = 0.39g, 39%. Rf = 0.55 100% hexane.
^1H (400MHz, $CDCl_3$) δ: 0.93 (6H, d, J = 10.52 Hz, H-3"), 1.89-1.96 (1H, m, H-2"), 2.58 (2H, d, J = 7.18 Hz, H-1"), 5.73 (1H, H-1), 5.89 (1H, H-1), 7.15 (2H, d, J = 8.10 Hz, H-2',6') 7.37 (2H, d, J = 8.07 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 22.75 (C-3"), 30.72 (C-2"), 45.69 (C-1"), 119.98 (C-1), 123.90 (q, J = 2.72, C-3), 128.52 (C-2'-6'), 129.71 (C3'-5'), 131.38 (C-1') 139.43 (q, J = 0.297 Hz, C-2), 143.17 (C-4') ; υ_{max} /cm^{-1} 1681.62 (C=C), 2956 (C-H).

HRMS calcd for $C_{13}H_{15}F_3$ (M+) 228.25730; found 228.25741.

5.12.33 3,3,3-Trifluoro-2-(4-isobutylphenyl)propan-1-ol (**147**)



To 1-isobutyl-4-(3,3,3-trifluoroprop-1-en-2-yl)benzene (**146**) (0.457g, 2mmol) in DME (5ml) was added a 90% solution of borane dimethylsulfide (0.23g, 0.316ml, 1.5mmol) under N_2. This was allowed to reflux overnight. This solution was then cooled to 0°C to which a 50% NaOH solution was added followed by H_2O_2 (30% solution in H_2O). This was allowed to stir at that temperature for 30 minutes. The solution was then quenched with H_2O (10ml) and extracted with Et_2O (3 x 20ml). The combined organic layers were then dried over Na_2SO_4, concentrated *in vacuo* and chromatographed on silica gel using 100% Et_2O to afford the title compound as a pale yellow oil. Yield 0.34g, 69%. Rf = 0.72, 100% Et_2O.

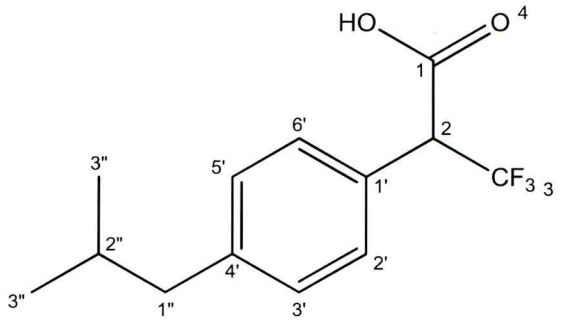
^{1}H (400MHz, $CDCl_3$) δ: 0.93 (6H, d, *J* = 10.52 Hz, H-3"), 1.89-1.96 (1H, m, H-2"), 2.47 (2H, d, *J* = 2.79 Hz, H-1"), 3.50, (1H, m, H-2) 3.97 (1H, m, H-1), 4.13, (1H, m, H-1), 7.15 (2H, d, *J* = 8.10 Hz, H-2',6') 7.37 (2H, d, *J* = 8.07 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 22.78 (C-3"), 30.54 (C-2"), 45.52 (C-1"), 52.49 (q, *J* = 0.253, C-2), 61.72 (C-1), 125.79 (q, *J* = 2.79, C-3), 126.19 (C-2',6'), 129.24 (C-3',5') 130.05 (C-1'), 142.70 (C-4').

υ_{max} /cm^{-1} 3289.96 (C-OH).

HRMS calcd for $C_{13}H_{17}F_3O$ (M+) 246.27258; found 246.27271.

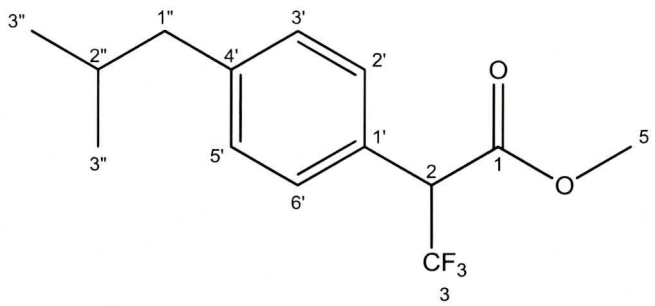
5.12.34 3,3,3-Trifluoro-2-(4-isobutylphenyl)propanoic acid[142] (**134**)



To chromium trioxide (0.32g, 3.248mmol) in acetone (11ml) was added 1.5M H_2SO_4 (5.4ml, 8.12mmol) at 0°C. To this was added compound **147** (0.2g, 0.812mmol) (predissolved in 1ml acetone). This was then allowed to warm to room temperature and stirred overnight. This was then diluted with Et_2O (10ml) and washed with brine (10ml). The organic layer was then extracted with 1M NaOH (2 x 10ml) followed by acidification of the aqueous layer with 1.5M H_2SO_4 to pH 1/2. The product was then extracted into ether (3 x 20ml) and the combined organic layers were then dried over Na_2SO_4, concentrated *in vacuo* and chromatographed on silica gel using 100 Et_2O to afford the title compound as colourless solid. Yield 0.207g, 99%. Rf = 0.34, 100% Et_2O;

^{1}H (400MHz, $CDCl_3$) δ: 0.83 (6H, d, *J* = 10.52 Hz, H-3"), 1.74-1.82 (1H, m, H-2"), 2.37 (2H, d, *J* = 4.35 Hz, H-1"), 4.23, (1H, q, *J* = 8.41, H-2) 7.08 (2H, d, *J* = 8.16 Hz, H-2',6') 7.26 (2H, d, *J* = 8.04 Hz, H-3',5'). ^{13}C (100MHz, $CDCl_3$) δ: 22.78 (C-3"), 30.54 (C-2"), 45.52 (C-1"), 52.49 (q, *J* = 0.253, C-2), 61.72 (C-2), 125.79 (q, *J* = 2.79, C-3), 126.19 (C-2',6'), 129.24 (C-3',5') 130.05 (C-1'), 142.70 (C-4'). 19F (400MHz) -68.15 (CF_3).υ_{max} /cm^{-1} 3400 (C-OH). (CI) m/z for $C_{13}H_{18}F_3O_2N$ ($M+NH_4^+$): 278 (100.00)

5.12.35 methyl 3,3,3-trifluoro-2-(4-isobutylphenyl)propanoate (**148**)



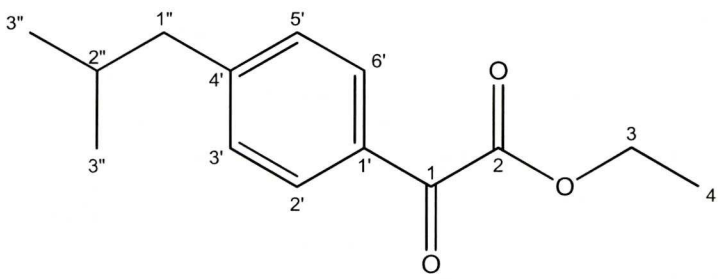
β-trifluoroibuprofen (**134**) (0.1g, 0.39mmol) in MeOH (1ml) was added conc H_2SO_4 (1 drop) and allowed to reflux for 4 hours. After which time the solvent was removed under reduced pressure and the dark yellow oil was purified by flash column chromatography using 100% Et_2O to afford the title compound as pale yellow oil. Yield 0.094g, 89%. Rf = 0.69, 100% Et_2O.

^1H (400MHz, $CDCl_3$) δ: 0.83 (6H, d, *J* = 10.52 Hz, H-3"), 1.74-1.82 (1H, m, H-2"), 2.37 (2H, d, *J* = 4.35 Hz, H-1"), 4.23, (1H, q, *J* = 8.41, H-2) 7.08 (2H, d, *J* = 8.16 Hz, H-2',6') 7.26 (2H, d, *J* = 8.04 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 22.78 (C-3"), 30.54 (C-2"), 45.52 (C-1"), 52.49 (q, *J* = 0.253, C-2), 61.72 (C-5), 125.79 (q, *J* = 2.79, C-3), 126.19 (C-2',6'), 129.24 (C-3',5') 130.05 (C-1'), 142.70 (C-4').

υ_{max} /cm^{-1} 3400 (C-OH).

5.12.36 Ethyl 2-(4-isobutylphenyl)-2-oxoacetate (**154**)



To a suspension of $AlCl_3$ (5.95g, 44.62mmol) in dry DCM (30ml) at 0°C was added chlorooxoacetate (6.1g, 5ml, 44.7mmol) dropwise. After 10 minutes at this temperature isobutylbenzene (2.98g, 3.5ml, 22.24mmol) was then added dropwise. This was allowed to

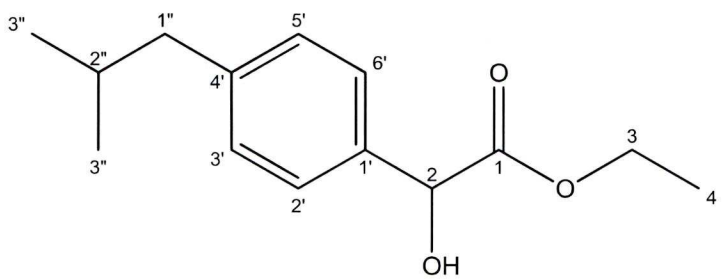
stir at 0°C for 1 hour. This was then poured onto ice and acidified with conc HCl to pH 1, extracted with Et_2O (2 x 50ml) and washed with 1M NaOH (40ml) and brine (50ml). The organic layers were dried over Na_2SO_4 and the resulting yellow oil was purified by flash column chromatography to yield the titled compound as pale yellow oil. Yield 3.70g, 71%. Rf = 0.68, 7:3 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 0.88 (6H, d, *J* = 6.64, H-3"), 1.39 (3H, t, *J* = 7.12, H-4), 1.90 (1H, m, H-2"), 2.52 (2H, d, *J* = 7.21, H-1"), 4.45 (1H, q, *J* = 7.11, H-3), 7.26 (2H, d, *J* = 8.16, H-3',5'), 7.90 (2H, d, *J* = 8.20, H-2',6').

^{13}C (100MHz, $CDCl_3$) δ: 14.46 (C-4), 22.65 (C-3"), 30.37 (C-2"), 45.81 (C-1"), 62.62 (C-3), 126.43 (C-2',6'), 129.62-132.70 (C-3'-5'), 150.80 (C-4') 164.50 (C-2), 186.59 (C-1); υ_{max} /cm^{-1} 2962.13 (C-H), 1733.69 (C=O), 1618.18 (C=O); (CI).

m/z for $C_{14}H_{21}O_3N$ ($M+NH_4^+$): 252 (100.00).

5.12.37 Ethyl 2-hydroxy-2-(4-isobutylphenyl)acetate (**155**)



To compound **154** (2g, 8.54mmol) in ethanol (10ml) at 0°C was added $NaBH_4$ (0.32g, 8.54mmol) portionwise. This was allowed to stir at that temperature for an hour. After which time the solvent was removed. Et_2O (20ml) was then added along with 1M HCl (10ml). The organic layer was separated and washed with brine, dried over Na_2SO_4 and purified using flash column chromatography. Yield 1.79g, 89%. Rf = 0.55, 7:3 Et_2O:hexane.

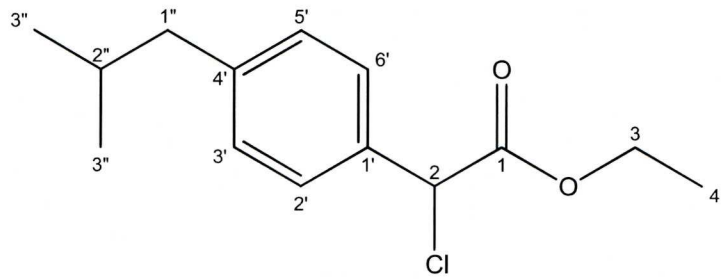
^1H (400MHz, $CDCl_3$) δ: 0.88 (6H, d, *J* = 6.62, H-3"), 1.39 (3H, t, *J* = 1.73, H-4), 1.90 (1H, m, H-2"), 2.45 (2H, d, *J* = 7.12, H-1"), 3.70 (1H, d, *J* = 5.89, OH), 4.19 (1H, dq, *J* = 17. 27, 10.48, H-3), 5.15 (1H, s, H-2) 7.11 (2H, d, *J* = 8.08, H-3',5'), 7.31 (2H, d, *J* = 8.08, H-2',6')

^{13}C (100MHz, $CDCl_3$) δ: 14.42 (C-4), 22.75 (C-1), 30.45 (C-2"), 45.53 (C-1"), 62.40 (C-3), 73.25 (C-2) 126.43 (C-3',5'), 129.60 (C-2',6'), 136.28 (C-1'), 142.29 (C-4'), 174.19 (C-1);

υ_{max} /cm^{-1} 3421.10 (OH), 2958.27 (C-H), 1708.62 (C=O).

(CI) m/z for $C_{14}H_{23}O_3N$ ($M+NH_4^+$): 254 (100.00).

5.12.38 Ethyl 2-chloro-2-(4-isobutylphenyl)acetate (**156**)



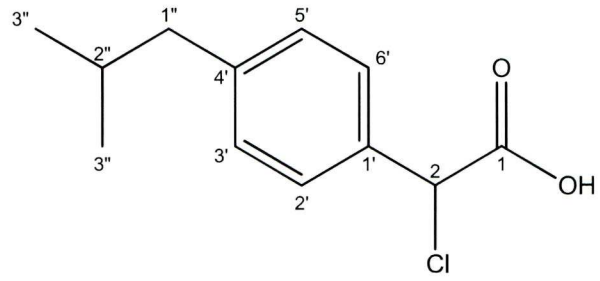
A solution of compound **155** (1g, 4.23mmol) in dry DCM (10ml) was reacted according to the procedure described in Section 5.11.27 using thionyl chloride (5g, 3.1ml, 42.3mmol) and a reaction time of 2 hours. The crude compound was purified by column chromatography using an eluent system of 1:9 Et_2O:hexane. Yield 0.97g, 90%. Rf = 0.54, 2:8 Et_2O:hexane;

^1H (400MHz, $CDCl_3$) δ: 0.89 (6H, d, *J* = 6.64, H-3"), 1.26 (3H, t, *J* = 7.17, H-4), 1.87 (1H, m, H-2"), 2.47 (2H, d, *J* = 7.21, H-1"), 4.23 (1H, m, H-3), 5.32 (1H, s, H-2), 7.15 (2H, d, *J* = 8.07, H-3',5'), 7.40 (2H, d, *J* = 8.16, H-2',6')

^{13}C (100MHz, $CDCl_3$) δ: 14.42 (C-4), 22.75 (C-3"), 30.45 (C-2"), 45.53 (C-1"), 60.10 (C-2), 62.39 (C-3), 128.22 (C-3',5'), 130.10 (C-2',6'), 136.28 (C-1'), 142.29 (C-4'), 174.19 (C-1);

HRMS calcd for $C_{14}H_{19}O_2{}^{35}ClNa$ (M+Na) 277.0971; found 277.0965. HRMS calcd for $C_{14}H_{19}O_2{}^{37}ClNa$ (M+Na) 277.0942; found 277.0950

5.12.39 2-Chloro-2-(4-isobutylphenyl)acetic acid (**157**)



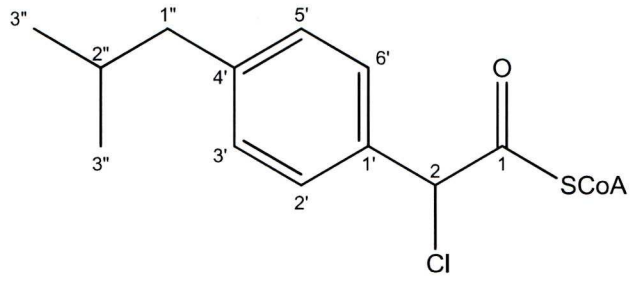
To compound **156** (0.5g, 1.96mmol) in acetic acid (2ml) and concentrated hydrochloric acid (3ml), was heated under reflux for 1.5 hours. The solvent was then removed under reduced pressure. The residue was allowed to cool to room temperature and poured into saturated sodium bicarbonate solution (10ml) at 0°C. The aqueous layer was then washed with ether (2 x 10ml). The aqueous phase was then acidified with dropwise addition of 5M HCl to pH 1-2. The orange precipitate was then extracted into ether (3 x 10ml), washed with water (2 x 10ml) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the resulting dark solid was purified by column chromatography using an eluent system of 1:1 Et_2O:hexane to yield the title compound as an orange solid. Yield 0.32g, 73%. Rf = 0.15, 7:3 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 0.89 (6H, d, *J* = 6.58 Hz, H-3"), 1.80-1.90 (1H, sept,*J* = 6.71 Hz H-2"), 2.46, (2H, d, *J* = 7.16 Hz H-1"), 5.54 (1H, s, H-2) 7.16 (2H, d, *J* = 8.10 Hz, H-2',6') 7.40 (2H, d, *J* = 8.17 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 22.78 (C-3"), 30.56 (C-2") 45.52 (C-1"), 59.13 (C-2), 128.21 (C-2',6'), 130.12 (C-3',5'), 132.67 (C-1') 143.91 (C-4') 174.81 (C-1).

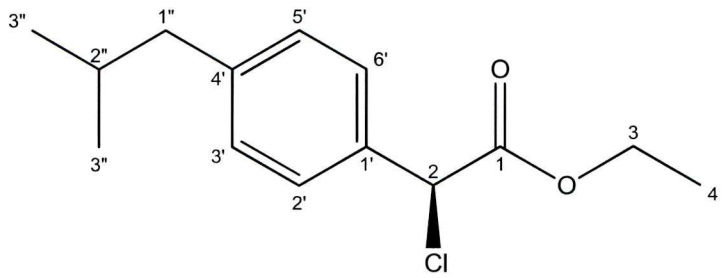
HRMS calcd for $C_{12}H_{15}O_2{}^{35}ClNa$ (M+Na) 249.0658; found 249.0647. HRMS calcd for $C_{12}H_{15}O_2{}^{37}ClNa$ (M+Na) 251.0629; found 251.0623.

5.12.40 2-Chloro-2-(4-isobutylphenyl)acetoyl-CoA (**64**)



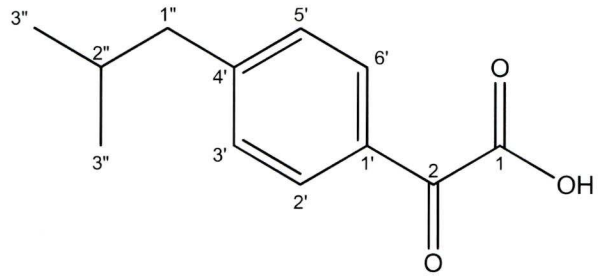
α-Chloroibufenac (**157**) (20mg, 88.24μmol) was reacted according to the procedure described in Section 5.11.6 to afford the title compound as a crystalline solid (2.15mg, 25%) based on relative HPLC traces of coenzyme A and product; HRMS calcd for $C_{33}H_{48}{}^{35}ClN_7O_{17}P_3S$ 974.1729 ($M+H^-$); found 974.1732.

5.12.41 (*S*)-α-Chloro-ibufenac (**156a**)



To 0.1M potassium phosphate buffer (pH 7, 1ml) was added *Candida rugosa* (15mg) followed by α-chloro-ibufenac ethyl ester (**156**). This was stirred at room temperature for 5 hours. After which time 100μL was removed and extracted with Et_2O (100μl). The ether layer was subject to HPLC analysis to ascertain extent of chiral hydrolysis

5.12.42 2-(4-Isobutylphenyl)-2-oxoacetic acid (**213**)



To a solution of 2*N* sodium hydroxide (2ml) in MeOH (5ml) was added compound **154** (0.5g, 2.11mmol). This was allowed to stir at room temperature for 2 hours. After which time the solution was diluted with water (10ml) and the methanol was removed under reduced pressure. The aqueous solution was washed with diethyl ether (2 x 10ml) and acidified with 2M HCl to which a white precipitate formed. This was extracted into ether (3 x 10ml), dried over $MgSO_4$ and evaporated to dryness to yield the desired compound as a white solid. Yield = 0.44g, 73%. Rf = 0.26, 7:3 Et_2O:hexane.

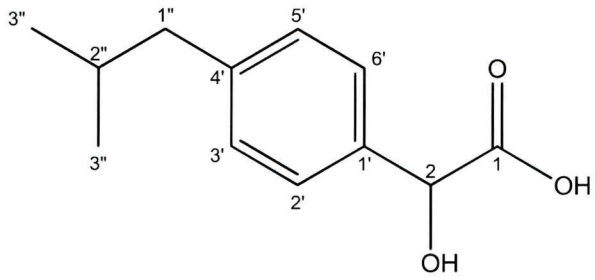
^1H (400MHz, $CDCl_3$) δ: 0.91 (6H, d, *J* = 6.63, H-3"), 1.92 (1H, sept, *J* = 6.70, H-2"), 2.56 (2H, d, *J* = 7.22, H-1") 7.29 (2H, d, *J* = 8.25, H-3',5') 8.16 (2H, d, *J* = 8.31, H-2',6')

^{13}C (100MHz, $CDCl_3$) δ: 22.74 (C-3"), 30.58 (C-2"), 45.51 (C-1"), 129.94 (C-3',5'), 131.32 (C-2',6'), 135.52 (C-1'), 151.17 (C-4'), 164.23 (C-1), 185.07 (C-2);

υ_{max} /cm^{-1} 2979.48 (C-H), 1677.77 (C=O) 1604.48 (C=O).

HRMS calcd for $C_{12}H_{14}O_3Na$ (M+Na) 229.0840 found; 229.0836.

5.12.43 2-Hydroxy-2-(4-isobutylphenyl)acetic acid (**214**)



Ethyl-2-hydroxy-2-(4-isobutylphenyl)acetate (**155**) (0.5g, 2.12mmol) was reacted in accordance with the procedure stated in Section 5.11.42 to yield the desired compound as a white solid. This was then purified by flash column chromatography using an eluent system of 7:3 Et_2O:hexane. Yield 0.41g, 92%. Rf = 0.18, 7:3 Et_2O:hexane.

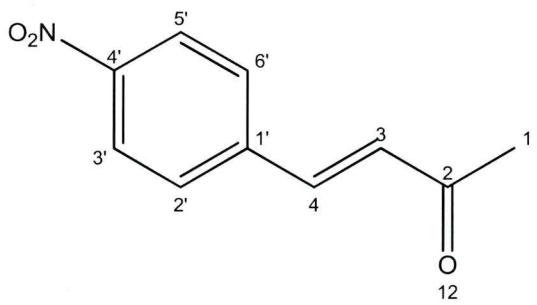
^1H (400MHz, $CDCl_3$) δ: 0.89 (6H, d, *J* = 6.62, H-3"), 1.84 (1H, sept *J* = 6.70, H-2"), 2.46 (2H, d, *J* = 7.18, H-1") 4.64 (1H, s, OH), 5.17 (1H, s, H-2), 7.13 (2H, d, *J* = 8.03, H-3',5') 7.32 (2H, d, *J* = 8.03, H-2',6').

^{13}C (100MHz, $CDCl_3$) δ: 22.75 (C-3"), 30.58 (C-2"), 45.51 (C-1"), 72.99 (C-2) 126.83 (C-3',5'), 129.85 (C-2',6'), 135.52 (C-1'), 142.77 (C-4'), 177.04 (C-1).

υ_{max} /cm^{-1} 3515.6 (O-H), 2979.48 (C-H), 1693.19 (C=O).

HRMS calcd for $C_{12}H_{16}O_3Na$ (M+Na) 231.0997 found; 231.0989.

5.12.44 (*E*)-4-(4-Nitrophenyl)but-3-en-2-one (**167**)[179]



p-Nitrobenzaldehyde (5g, 33.09mmol) was dissolved in acetone (60ml) at 0°C. An aqueous solution of NaOH (6ml, 1% solution) was added dropwise and allowed to stir at that temperature for 15 minutes. The solution was acidified to pH 2 with dropwise addition of

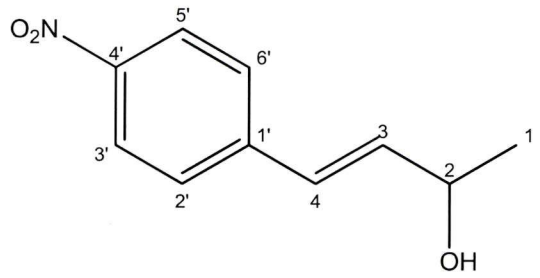
1.5M H_2SO_4 and the acetone was removed under reduced pressure. To the resulting crude 4-hydroxy-4-nitrophenyl-butan-2-one was added 35ml 1.5M H_2SO_4 and was heated to reflux for 2 hours. The resulting orange solid was then filtered off and recrystallized from ethanol to yield the title compound as a bright yellow solid. Yield = 5.88g, 93%. Rf = 0.55, 100% Et_2O.
^1H (400MHz, $CDCl_3$) δ: 2.43 (3H, s, H-1), 6.83 (1H, d, J = 16.25 Hz, H-3), 7.54, (1H, d, J = 16.34 Hz H-4), 7.71 (2H, d, J = 8.70 Hz, H-2',6') 8.27 (2H, d, J = 8.59 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 28.47 (C-1), 124.62 (C-3',5') 129.22 (C-2',6'), 130.78 (C-3) 140.48 (C-4), 141.07 (C-1'), 148.99 (C-4'), 197.96 (C-2).

υ_{max} /cm^{-1} = 2886.92 (C-H), 1710.55 (C=O) 1596.77 (C=C), 1513.85, 1344.14 (NO_2).

(Cl) m/z for $C_{10}H_8NO_3$ ($M-H^-$): 190.1 (100.00)

5.12.45 (*E*)-4-(4-Nitrophenyl)but-3-en-2-ol (**168**)

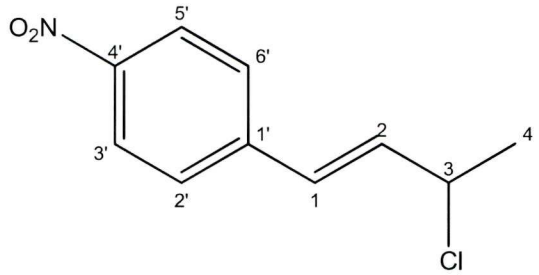


To a solution of compound **167** (3g, 15.69mmol) and $CeCl_3.7H_2O$ (5.84g, 15.69mmol) in MeOH (20ml) was added $NaBH_4$ (0.59g, 15.69mmol) in one portion, with stirring. A vigorous gas evolved, together with a temperature rise to ≈35-40°C. After 30 minutes the pH was adjusted to 7 with 1M HCl. The mixture was then extracted with Et_2O (2 x 20ml) and dried over Na_2SO_4. The solvent was then removed under reduced pressure and purified with flash column chromatography to yield a yellow solid. Yield 2.606g, 86%. Rf = 0.48, 100% Et_2O.
^1H (400MHz, $CDCl_3$) δ: 1.41 (3H, d, J = 6.44 Hz, H-1), 4.56 (1H, m, H-2) 6.45 (1H, dd, $J_{H2\text{-}H3}$ = 5.67Hz, $J_{H3\text{-}H4}$ = 15.90, H-3), 6.66, (1H, d, J = 16.01 Hz H-4), 7.50 (2H, d, J = 8.84 Hz, H-2',6') 8.16 (2H, d, J = 8.85 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 23.76 (C-1), 68.77, (C-2), 124.39 (C-3',5') 127.37 (C-2',6'), 127.38 (C-4) 138.88 (C-3), 143.79 (C-1'), 147.26 (C-4').

υ_{max} /cm^{-1} = 2884.99 (C-H), 1636 (C=C), 1517.7, 1346.07 (NO_2). HRMS calcd for $C_{10}H_{11}O_3Na$ (M+Na) 216.0637; found 216.0641.

5.12.46 (*E*)-1-(3-Chlorobut-1-en-1-yl)-4-nitrobenzene (**169**)

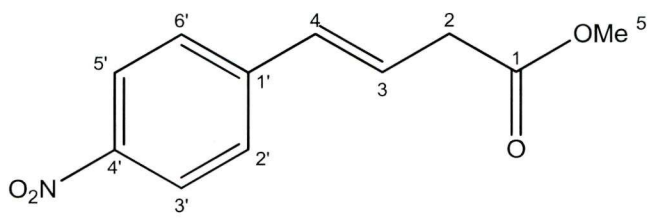


(*E*)-1-(3-chlorobut-1-en-1-yl)-4-nitrobenzene (**169**) was synthesised in the same manner as compound (**156**), using (*E*)-4-(4-nitrophenyl)but-3-en-2-ol **(168)** (1g, 5.18mmol), DCM (20ml) and thionyl chloride (6.15g, 3.75ml, 51.75mmol) to yield the title compound as a yellow solid. Yield 0.99g, 90%. Rf = 0.75, 100% Et_2O.

^{1}H (400MHz, $CDCl_3$) δ: 1.73 (3H, d, J = 6.65 Hz, H-4), 4.74 (1H, m, H-3) 6.46 (1H, dd, $J_{H1\text{-}H2}$ = 7.80Hz, $J_{H2\text{-}H3}$ = 15.69, H-2), 6.66, (1H, d, J = 15.76 Hz H-1), 7.53 (2H, d, J = 8.76 Hz, H-2',6') 8.20 (2H, d, J = 8.81 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 25.42 (C-4), 57.53, (C-3), 124.45 (C-3',5') 127.68 (C-2',6'), 129.16 (C-1) 135.92 (C-2), 142.84 (C-1'), 147.69 (C-4').
HRMS calcd for $C_{10}H_{10}O_2{}^{37}ClNa$ (M+Na) 236.0268; found 236.0274. HRMS calcd for $C_{10}H_{10}O_2{}^{35}ClNa$ (M+Na) 234.0298; found 234.0290.

5.12.47 (*E*)-Methyl 4-(4-nitrophenyl)but-3-enoate (**172**)[158]



A mixture of compound **167** (0.5g, 2.62mmol), methanol (0.25g, 0.32ml, 7.85mmol) and borontrifluoride etherate (3.22g, 31mmol, 1.97 ml) was added in one portion to a stirred suspension of lead(IV) acetate (1.34g, 3.01mmol) in dry toluene (10 ml) at room temperature.

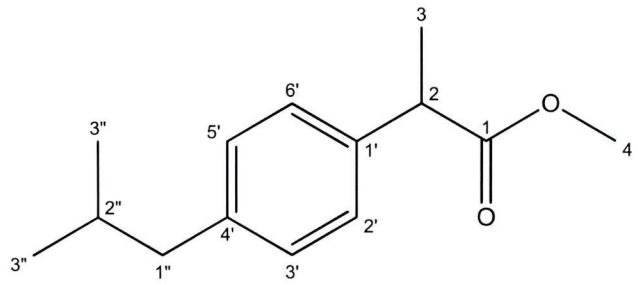
This was allowed to stir at room temperature for 1 hour after which time it was washed with saturated sodium hydrogen carbonate (2 x 10ml), followed by brine (10ml). The organic layer was then dried over Na_2SO_4 and evaporated to dryness to yield the crude material as dark brown oil. This was then purified by flash column chromatography to yield the title compound as a pale yellow oil. Yield 0.32g, 55%. Rf = 0.12, eluent: 7:3 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 3.33 (2H, d, *J* = 5.97 Hz, H-2), 3.74 (3H, s, H-5) 6.47-6.60 (2H, m, H-3,4), 7.51 (2H, d, *J* = 8.87 Hz, H-2',6') 8.16 (2H, d, *J* = 8.87 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 38.49 (C-2), 52.51, (C-5), 124.37 (C-2',6') 127.23 (C-3',5'), 127.28 (C-4), 131.13 (C-3) 143.60 (C-1'), 147.31 (C-4'), 171.70 (C-1). υ_{max} /cm^{-1} 2979.48 (C-H), 1729.83 (C=O), 1596.77 (C=C), 1517.7, 1344.14 (NO_2).

HRMS calcd for $C_{11}H_{10}NO_4(M\text{-}H^+)$ 220.0603; found 220.0610.

5.12.48 Methyl 2-(4-isobutylphenyl)propanoate[180] (**176**)

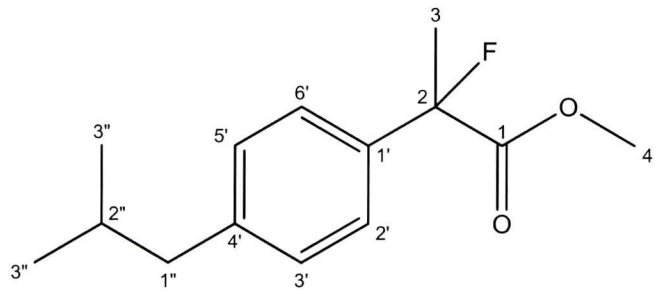


Ibuprofen (**175**) (1g, 4.85mmol) in methanol (20ml) was reacted according to the procedure described in Section 5.11.41. The crude material was purified with flash column chromatography using an eluent system of 1:1 Et_2O:hexane to yield the title compound as a yellow oil. Yield 1.00g, 99%. Rf = 0.57, 1:1 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 0.82 (6H, d, *J* = 6.64 Hz, H-3"), 1.42 (3H, d, *J* = 7.18 Hz, H-3) 1.72-1.82 (1H, sept,*J* = 6.71 Hz H-2"), 2.37, (2H, d, *J* = 7.27 Hz H-1"), 3.56 (3H, s, H-4), 7.03 (2H, d, *J* = 8.09 Hz, H-2',6') 7.15 (2H, d, *J* = 8.09 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 13.76 (C-3) 21.96 (C-3"), 29.69, (C-2"), 30.58 (C-2) 45.44 (C-1"), 52.54 (C-4), 125.70 (C-2',6'), 129.76 (C-3',5'), 140.45 (C-1'), 142.31 (C-4') 177.85 (C-1). (Cl) m/z for $C_{14}H_{24}O_2N$ ($M + NH_4^+$): 238 (100.00),

5.12.49 Methyl 2-fluoro-2-(4-isobutylphenyl)propanoate[181] (**177**)

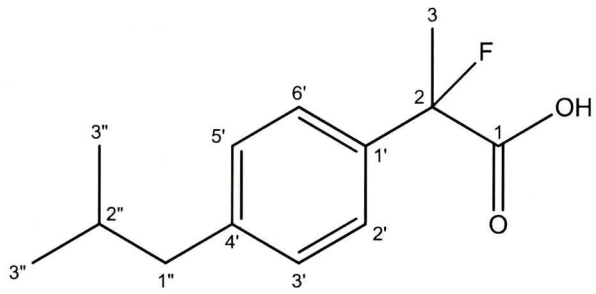


To compound **176** (0.67g, 3.04 mmol) in THF (20ml) at -78°C under N_2 was added 2M LDA (premade with 1.58ml of 2.5M n-BuLi and 0.56ml diisopropylamine predissolved in THF (5ml)) dropwise. This was allowed to stir at that temperature for 30 minutes. To this was then added *N*-fluorobenezesulfonimide (previously dissolved in a minimum amount of THF) dropwise. This was allowed to stir at that temperature for 1 hour. The reaction was then quenched with saturated ammonium chloride (20ml) and diluted with Et_2O (20ml). The aqueous layer was separated and the organic layer was washed with brine (20ml), followed by H_2O (2 x 20ml). The organic layer was then dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was then purified using flash column chromatography using an eluent system of 1:4 EtOAc:hexane to yield the title compound as a colourless oil. Yield = 0.47g, 66%. Rf = 0.57, 1:1 Et_2O:hexane.

^{1}H (400MHz, $CDCl_3$) δ: 0.81 (6H, d, *J* = 6.71 Hz, H-3"), 1.73-1.80 (1H, m, H-2"), 1.87, (3H, d, *J* = 22.12 Hz H-3) 2.38 (2H, d, *J* = 7.15 Hz, H-1"), 3.66 (3H, s, H-4), 7.07 (2H, d, *J* = 8.12 Hz, H-2',6') 7.31 (2H, d, *J* = 8.23 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 21.31 (C-3"), 23.68, (d, J = 23.78, C- 3), 29.11 (C-2") 43.97 (C-1"), 51.80 (C-4), 93.57 (d, J = 185.33 (C-2), 123.33 (d, *J* = 8.17, C-2',6'), 128.18 (C-3',5'), 135.37 (d, *J* =22.79, C-1') 141.29 (C-4') 170.52 (d, *J* = 27.27, C-1).
HRMS calcd for $C_{14}H_{19}O_2FNa$ (M+Na) 261.1267; found 261.1258.

5.12.50 2-Fluoro-2-(4-isobutylphenyl)propanoic acid[182] (**178**)



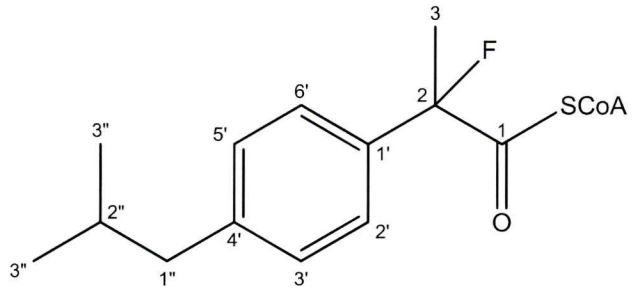
To compound **177** (0.3g, 1.26mmol) in a mixture of methanol (3ml) and THF (2ml) was added 2 equivalents of 1M KOH (2ml) dropwise. This was allowed to stir at room temperature for 3 hours. After which time methanol and THF were removed under reduced pressure. The residual aqueous solution was diluted with H_2O (5ml) and extracted with Et_2O (2 x 10ml). The aqueous layer was then acidified with 1M HCl to pH 2/3 which resulted in the precipitation of a white solid. This was then washed with Et_2O. The organic layer was dried over Na_2SO_4 and removed under reduced pressure to yield the desired compound as white solid. Yield 0.27g, 96%. Rf = 0.32, 1:1 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 0.95 (6H, d, *J* = 5.90 Hz, H-3"), 1.83-1.99 (1H, m, H-2"), 2.00, (3H, d, *J* = 22.12 Hz H-3) 2.67 (2H, d, *J* = 7.15 Hz, H-1"), 7.22 (2H, d, *J* = 7.31 Hz, H-2',6') 7.49 (2H, d, *J* = 7.41 Hz, H-3',5'), 11.60 (1H, s, OH).

^{13}C (100MHz, $CDCl_3$) δ: 22.80 (C-3"), 24.68, (d, *J* = 23.44, C- 3), 30.59 (C-2") 45.45 (C-1"), 94.48 (d, *J* = 185.60 (C-2), 125.01 (C-2',6'), 129.76 (C-3',5'), 136.01 (d, *J* =22.92 Hz, C-1') 143.14 (C-4') 177.72 (d, *J* = 28.33, C-1) ; υ_{max} /cm^{-1} 3509.09 (OH), 2956.34 (C-H), 1731.76 (C=O).

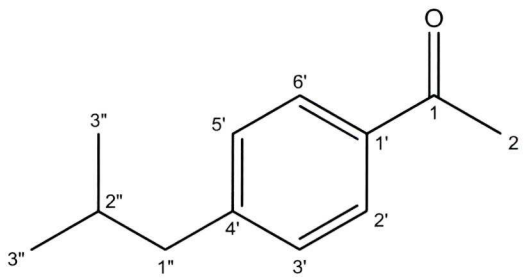
HRMS calcd for $C_{13}H_{17}O_2FNa$ (M+Na) 247.1110; found 247.1099.

5.12.51 α–Fluoroibuprofenoyl-CoA (**62**)



α-Fluoroibuprofen (**178**) (50mg, 0.22mmol) and CoA-SH, $(Li^{+})_3$ (17mg, 21.68μmol) were reacted according to the procedure described in Section 5.11.6. to afford the title compound as a crystalline solid (4.64mg, 22%) based on relative HPLC traces of coenzyme A and product; HRMS calcd for $C_{34}H_{50}FN_7O_{17}P_3S$ ($M+H^{-}$) 972.2181; found 972.2163

5.12.52 *p*-Isobutylacetophenone[183] (**179**)



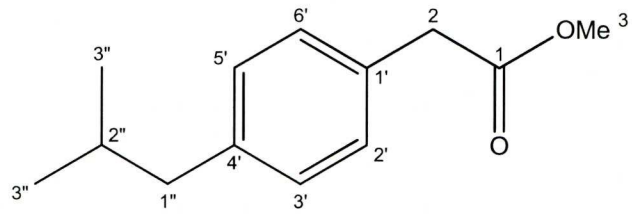
Isobutylbenzene (4.5ml, 29mmol) was reacted in accordance to Section 5.11.5 using $AlCl_3$ (8.5g, 63.8mmol), DCM (20ml) and acetic anhydride (2.74ml, 29mmol) with a reaction time of 2 hours. The crude material was chromatographed on silica gel using 100% hexane to afford the title compound as a pale yellow oil. Yield 6.3g, 74%. Rf = 0.43, 100% :hexane.

^{1}H (400MHz, $CDCl_3$) δ: 0.91 (6H, d, J = 6.59 Hz, H-3"), 1.91 (1H, sept, J = 6.77, H-2"), 2.52, (1H, s, H-2) 2.55 (2H, d, J = 7.22 Hz, H-3',5'), 7.27 (2H, d, J = 8.43 Hz, H-2',6') 7.93 (2H, d, J = 8.40 Hz, H-5,9).

^{13}C (100MHz, $CDCl_3$) δ: 22.71 (C-3"), 26.85, (C-2), 30.50 (C-2") 45.95 (C-1"), 129.85 (C-1') 130.04 (C-2',6'), 130.70 (C-3',5'), 149.98 (C-4') 201.36 (C-1'). υ_{max} /cm^{-1} 2979.48 (C-

H), 1681.62 (C=O). No MS data obtained. NMR data synomous with previously published work.

5.12.53 Methyl 2-(4-isobutylphenyl)acetate (**180**)

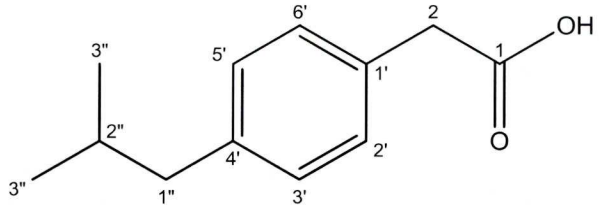


Sulfuric acid (0.37ml, 6.8mmol) was added to a stirred solution of compound **179** (1g, 5.67mmol) and diacetoxyiodobenzene (2.19g, 6.8mmol) in 15ml MeOH at room temperature under N_2. The reaction was stirred for 5 hours at 60°C, quenched with the addition of water (10ml), and extracted with ether (2 x 10ml). This was washed with H_2O (2 x 10ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure and purified by column chromatography using an eluent system of 1:1 Et_2O:hexane to yield the titled compound as a pale yellow oil. Yield 0.73g, 63%. Rf = 0.55, eluent: 7:3 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 0.89 (6H, d, *J* = 6.59 Hz, H-3"), 1.78-1.89 (1H, sept, *J* = 6.70 H-2"), 2.44 (2H, d, *J* = 7.14 Hz, H-1"), 3.57 (2H, s, H-2), 3,65 (3H, s, H-3) 7.08 (2H, d, *J* = 8.05 Hz, H-2',6'), 7.17 (2H, d, *J* = 7.99 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 22.80 (C-3"), 30.62 (C-2") 40.21(C-2) 45.51 (C-1"), 52.31 (C-3), 129.38 (C-3',5'), 129.74 (C-2',6'), 131.67 (C-1'), 140.90 (C-4') 172.58 (C-1) υ_{max} /cm^{-1} 2950.55 (C-H), 1737.55 (C=O).
HRMS calcd for $C_{13}H_{18}O_2Na$ (M+Na) 229.1205; found 229.1201

5.12.54 2-(4-Isobutylphenyl)acetic acid[184] (**181**)



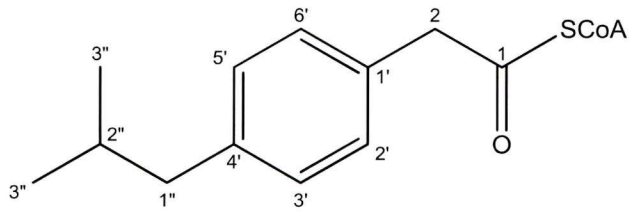
Compound (**180**) (0.5g, 2.42mmol) was used to synthesise (**181**) in the same manner as described in Section 5.11.42. This was purified by flash column chromatography using an

eluent system of 1:1 Et_2O:hexane to yield the titled compound as a white solid. Yield 0.44g, 94%. Rf = 0.42, eluent: 7:3 Et_2O:hexane.

^1H (400MHz, $CDCl_3$) δ: 0.89 (6H, d, *J* = 6.57 Hz, H-3"), 1.91 (1H, sept, *J* = 6.69 H-2"), 2.44 (2H, d, *J* = 7.13 Hz, H-1"), 3.68 (2H, s, H-2), 7.25 (2H, d, *J* = 7.95 Hz, H-2',6'), 7.17 (2H, d, *J* = 7.95 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 22.74 (C-3"), 30.54 (C-2") 41.13 (C-2) 45.47 (C-1"), 129.49 (C-3',5'), 129.94 (C-2',6'), 130.88 (C1'), 141.23 (C-4') 178.61 (C-1) υ_{max} /cm^{-1} 2950.55 (C-H), 1698.98 (C=O).
HRMS calcd for $C_{12}H_{16}O_2Na$ (M+Na) 215.1048; found 215.1050.

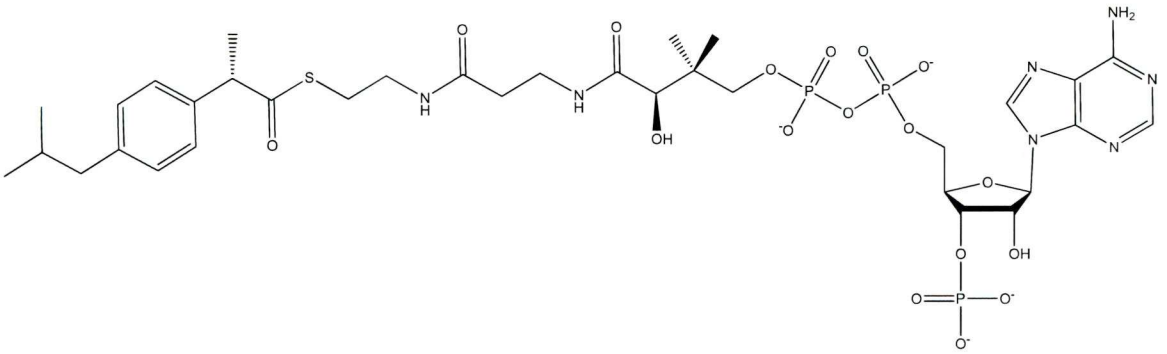
5.12.55 2-(4-Isobutylphenyl)acetoyl-CoA (**182**)



Ibufenac (**181**) (5mg, 0.26mmol) was reacted with CoA-SH, $(Li^+)_3$ (5mg, 6.4μmol) according to the procedure described in Section 5.11.6. to afford the title compound as a crystalline solid (1.03mg, 20%) based on relative HPLC traces of coenzyme A and product; HRMS calcd for $C_{33}H_{49}N_7O_{17}P_3S$ 940.2119 ($M+H^-$); found 940.2120

5.13 Synthesis of Substrates

5.13.1 Ibuprofenoyl-CoA (**22**)

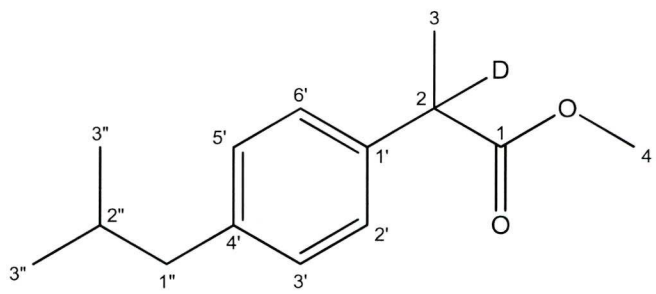


(*S*)-Ibuprofen (**175a**) (5mg, 24.24μmol) and CoA-SH, $(Li+)_3$ (20mg, 25μmol) was reacted according to the procedure described in Section 5.11.6. to yield the title compound as a crystalline white solid. Yield = 10.36mg, 43%.

^{1}H (400MHz, $CDCl_3$) δ: 0.70, (3H, s, H-13/14), 0.80 (6H, d, *J* = 6.60Hz, H-1), 0.85 (3H, s, H-13/14),1.44 (3H, d, *J* = 6.99Hz, H-7) 1.73 (1H, sept, *J* = 6.89Hz, H-2), 2.22 (2H, t, 6.38 Hz, H-10), 2.38 (2H, d, *J* = 7.02 Hz, H-3), 2.96 (2H, m, H-8), 3.28 (4H, m, H-9-11), 3.53 (2H, m, H-15), 3.82 (2H, m, H-16), 3.96 (1H, q, *J* = 7.02Hz, H-6), 3.99 (1H, s, H-12), 4.24 (2H, m, H-17-18), 4.57 (1H, m, H-19), 6.14 (1H, d, *J* = 6.47Hz , H-20), 7.11 (2H, d, *J* = 7.94Hz, H-5), 7.19 (2H, d, *J* = 7.94Hz, H4), 8.20 (1H, s, H-21), 8.54 (1H, s, H-22)

HRMS calcd for $C_{34}H_{51}N_7O_{17}P_3S$ ($M+H^-$) 954.2275; found 954.2465

5.13.2 α-Deutero-methyl 2-(4-isobutylphenyl)propanoate[185] (**183**)



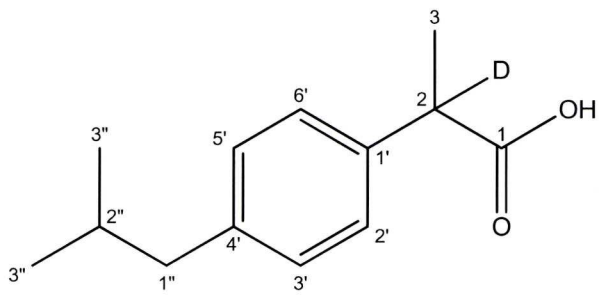
To compound **176** (0.8g, 3.88mmol) in CD_3OD (7ml) was added NaOMe (0.2g, 3.88mmol) and stirred under reflux for 20 hours. The reaction was stopped with the dropwise addition of 2M HCl at 0°C until a pH of 7 was obtained. This was then filtered through a pad of celite, concentrated, diluted with hexane (10ml), washed with H_2O (2 x10ml), dried over Na_2SO_4

and concentrated under reduced pressure. This was then purified by column chromatography using an eluent system of 1:1 Et_2O:hexane to yield the title compound as a yellow oil. Yield 0.8g, 99%. Rf = 0.57, 1:1 Et_2O:hexane;

^1H (400MHz, $CDCl_3$) δ: 0.90 (6H, d, *J* = 6.61 Hz, H-3"), 1.44 (3H, s, H-3) 1.79-1.89 (1H, sept, *J* = 6.76 Hz H-2"), 2.45, (2H, d, *J* = 7.21 Hz H-1"), 3.37 (3H, s, H-4), 7.09 (2H, d, *J* = 8.26 Hz, H-2',6') 7.19 (2H, d, *J* = 8.25 Hz, H-3',5');

^{13}C (100MHz, $CDCl_3$) δ: 18.93 (C-3), 22.81 (C-3"), 30.60 (C-2"), 45.47 (C-1"), 127.66 (C-2',6'), 129.77 (C-3',5'), 138.11 (C-1'), 140.97 (C-4'), 175.69 (C-1). No MS data obtained. NMR data synonymous with previously published work.

5.13.3 α-Deutero-Ibuprofen (**183**)[185]

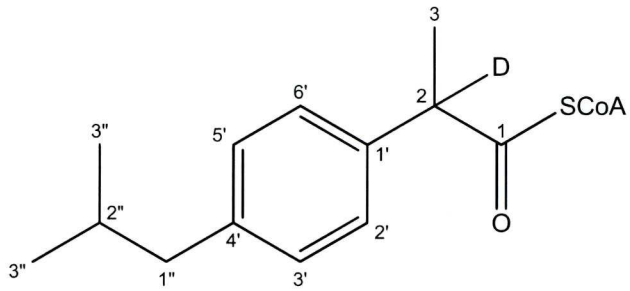


Sodium hydride, 60% dispersed in mineral oil (0.099g, 2.48mmol) was slowly added to a stirred solution of α-D-methyl-2-(4-isobutylphenyl)propanoate (**183**) (0.5g, 2.26mmol) in THF/D_2O (20 ml, 3:1) at room temperature. The resulting solution was stirred for 12hours at that temperature. The reaction was quenched with the addition of water (20 ml), and acidified with 5ml 1M HCl. This was extracted with DCM (2 x 10ml) and the combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure to give the title compound as a white solid. Yield 0.46g, 99%. Rf = 0.32, 1:1 Et_2O:hexane;

^1H (400MHz, $CDCl_3$) δ: 0.82 (6H, d, *J* = 6.64 Hz, H-3"), 1.42 (3H, s, H-3) 1.72-1.82 (1H, sept,*J* = 6.71 Hz H-2"), 2.37, (2H, d, *J* = 7.27 Hz H-1"), 7.03 (2H, d, *J* = 8.09 Hz, H-2',6') 7.15 (2H, d, *J* = 8.09 Hz, H-3',5');

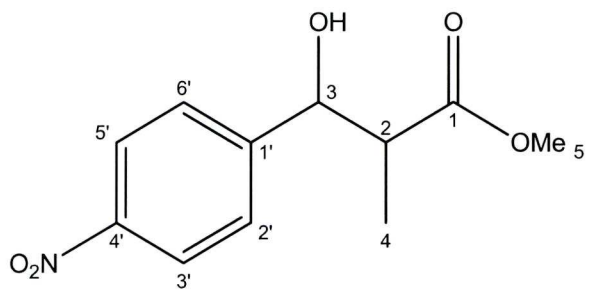
HRMS calcd for $C_{13}H_{17}O_2Na$ (M+Na) 230.1267; found 230.1276.

5.13.4 α-Deutero-Ibuprofenoyl-CoA (**67**)



α-D-Ibuprofen (**220**) (5mg, 24.12μmol) was reacted according to the procedure described in Section 5.11.6 to afford the title compound as a crystalline solid. Yield = 4.61mg, 20%; HRMS calcd for $C_{34}H_{53}N_7O_{17}P_3SD$ $(M\text{-}2H)^{2-}$ 477.1135; found 477.1128

5.13.5 Methyl 3-hydroxy-2-methyl-3-(4-nitrophenyl)propanoate (**186**)



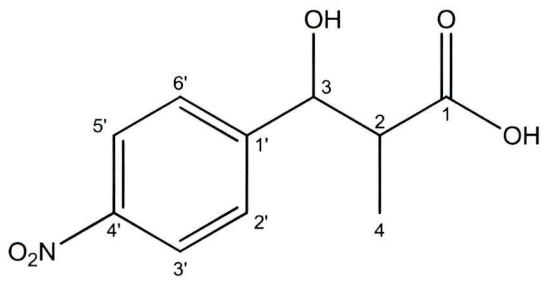
To 2.198ml (22.8mmol) propionic acid methyl ester in 200ml DCM was added dibutyl boron triflate (29.64ml of 1M solution in DCM, 29.64mmol) and diisopropyl ethyl amine (4.42g, 5.95ml, 34.2mmol) at 0°C. This was allowed to stir at that temperature for 30 minutes. After which time the reaction temperature was reduced to -78°C and 4-nitrobenzaldehyde was added (predissolved in 30ml DCM). This was allowed to stir at that temperature for 30 minutes and then at room temperature for 2 hours. The reaction mixture was then quenched with potassium phosphate buffer (pH7, 100ml) and extracted with ether (2 x 100ml). The extract was then washed with 2*N* HCl (100ml), saturated sodium carbonate (100ml) and finally brine (100ml). The organic layers were dried over Na_2SO_4, evaporated to dryness under reduced pressure and purified by column chromatography using 1:1 Et_2O:hexane to yield the title compound as a pale yellow solid. Yield 3.93g, 72%. Rf = 0.32, 7:3 Et_2O:hexane.

^{1}H (400MHz, $CDCl_3$) δ: 1.08 (3H, d, *J* = 7.32 Hz, H-4), 2.82 (1H, dq *J* = 7.2, 3.6, H-2), 3.72 (3H, d, *J* = 6.16 Hz, H-5), 5.25, (1H, d, *J* = 3.48, H-3) 7.53 (2H, d, *J* = 8.72 Hz, H-2',6') 8.21 (2H, d, *J* = 8.84 Hz, H-3','5').

^{13}C (100MHz, $CDCl_3$) δ: 10.62 (C-4), 46.14 (C-2), 52.59 (C-5), 72.87 (C-3), 123.93 (C-3',5'), 127.25 (C-2',6'), 127.86 (C-4'), 149.01 (C-1') 176.419 (C-1).

υ_{max} /cm^{-1} 3484.74 (OH), 2956.7 (C-H), 1708.82 (C=O), 1513.85 (NO_2), 1342.21 (NO_2).

5.13.6 3-Hydroxy-2-methyl-3-(4-nitrophenyl)propanoic acid (**188**)



To compound **186** (0.5g, 2.09mmol) in MeOH (3ml) was added 2ml 5*N* KOH dropwise at 0°C and left to stir for 1 hour. After this time MeOH was evaporated under reduced pressure and the mixture was then washed with EtOAc (2 x 5ml) to remove any unreacted starting material. The aqueous layer was acidified with the dropwise addition of conc HCl to pH 2 and extracted with EtOAc (2 x 5ml). The organic layer was dried over Na_2SO_4 and evaporated to give the title compound as a pale yellow solid without need for further purification. Yield 0.33g, 70%. Rf = 0.4, 100% EtOAc.

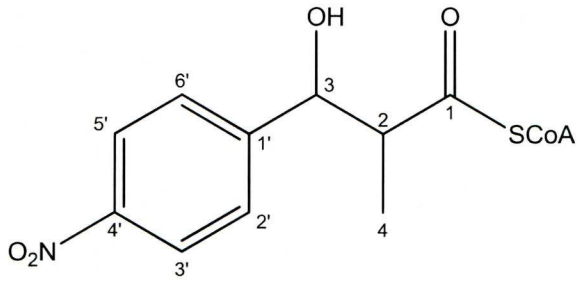
^{1}H (400MHz, $CDCl_3$) δ: 1.13 (3H, d, *J* = 7.32 Hz, H-4), 2.87 (1H, m, H-2), 5.34, (1H, d, *J* = 3.32, H-3) 7.53 (2H, d, *J* = 7.56 Hz, H-2',6') 8.25 (2H, d, *J* = 8.76 Hz, H-3',6').

^{13}C (100MHz, $CDCl_3$) δ: 10.30 (C-4), 46.19 (C-2), 72.68 (C-3), 124.04 (C-3',5'), 127.27 (C-2',6'), 131.74 (C-4'), 148.75 (C-1') 180.91 (C-1).

υ_{max} /cm^{-1} 3384.46 (OH), 1695.12 (C=O), 1511.92 (NO_2), 1344.14 (NO_2).

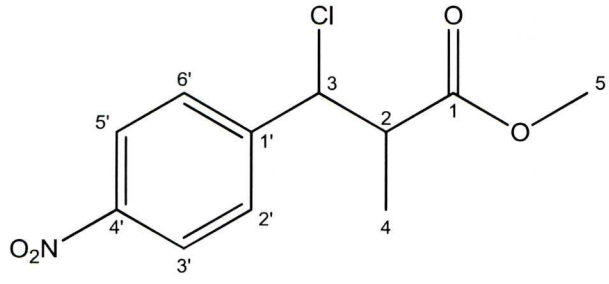
HRMS calcd for $C_{20}H_{20}N_2O_{10}Na$ $(2M+Na-2H)^-$ 471.1016; found 471.0990.

5.13.7 3-Hydroxy-2-methyl-3-(4-nitrophenyl)propanoyl-CoA (**68**)



3-hydroxy-2-methyl-3-(4-nitrophenyl)propanoic acid (**188**) (20mg, 88.81μmol) was reacted according to the procedure described in Section 5.11.6. to afford the title compound as a crystalline solid (1.56mg, 18%) based on relative HPLC traces of coenzyme A and product. HRMS calcd for $C_{31}H_{44}N_8O_{20}P_3S$ 973.1606 ($M+H^-$); found 973.1522.

5.13.8 Methyl 3-chloro-2-methyl-3-(4-nitrophenyl)propanoate (**167**)



To compound **186** (0.5g, 2.09mmol) in dry DCM (5ml) was added thionyl chloride (2.49g, 1.52ml, 21mmol). This was refluxed for 2 hours after which time the solvent was removed under reduced pressure to give a thick orange oil. This was purified by column chromatography using 100% Et_2O to afford the title compound as a pale yellow oil. Yield 0.41g,76%. Rf = 0.18, 7:3 Et_2O:hexane.

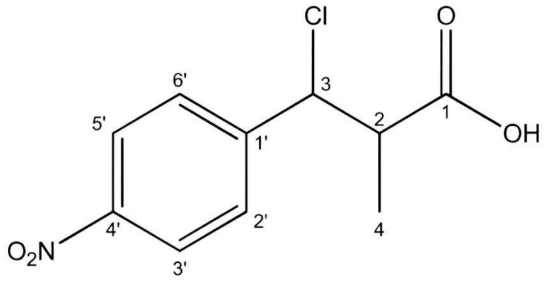
^1H (400MHz, $CDCl_3$) δ: 1.41 (3H, d, *J* = 6.93 Hz, H-4), 3.09 (1H, m, H-2), 3.57, (3H, s, H-5), 5.24 (1H, d, *J* = 8.25, 7.53, H-3) 7.59 (2H, d, *J* = 8.82 Hz, H-2',6') 8.21 (2H, d, *J* = 8.83 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 14.59 (C-4), 48.60 (C-5), 52.54 (C-2), 63.31 (C-3), 124.02 (C-3',5'), 128.60 (C-2',6'), 130.61 (C-1'), 147.58 (C-4') 173.06 (C-1).

υmax /cm^{-1} 2979.48 (C-H), 1733.69, 1521.56, 1344.14 (NO_2);

HRMS calcd for $C_{11}H_{12}N^{35}ClO_4Na$ (M+Na) 280.0353; found 280.0359.

5.13.9 3-Chloro-2-methyl-3-(4-nitrophenyl)propanoic acid (**189**)



To compound **167** (0.3g, 1.16mmol) in H_2O (2ml) was added H_2SO_4 (1ml) and the solvents allowed to reflux for 4 hours. This was then cooled to room temperature, extracted with Et_2O (2 x 5ml), dried over Na_2SO_4 and evaporated to dryness under reduced pressure. This was then purified by flash column chromatography using 7:3 Et_2O:hexane. Yield 0.28g, 99%. Rf = 0.32, eluent: 7:3 Et_2O:hexane.

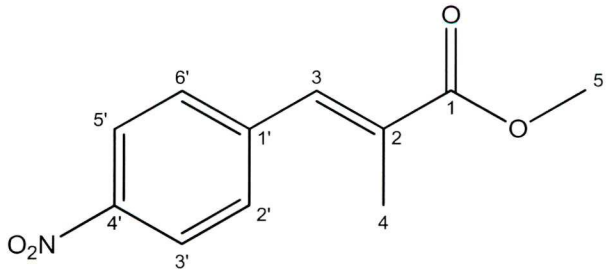
^1H (400MHz, $CDCl_3$) δ: 1.35 (3H, d, J = 10.60 Hz, H-4), 3.04 (1H, m, H-2), 5.21 (1H, d, J = 7.60 Hz, H-3) 7.67 (2H, d, J = 8.84 Hz, H-2',6') 8.15 (2H, d, J = 8.72 Hz, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 13.99 (C-4), 48.28 (C-2), 62.74 (C-3), 124.13 (C-3',5'), 128.86 (C-2',6'), 130.75 (C-1'), 146.68 (C-4') 178.32 (C-1).

υ_{max} /cm^{-1} 2979.48 (C-H), 1733.69 (C=O), 1521.56, 1344.14 (NO_2).

HRMS calcd for $C_{10}H_{10}NO_4{}^{35}Cl$ ($M+Na^+$) 266.0196 found 266.0188, HRMS calcd for $C_{10}H_{10}NO_4{}^{37}ClNa$ (M+Na) 268.0167 found 268.0176.

5.13.10 (*E*)-Methyl 2-methyl-3-(4-nitrophenyl)acrylate (**193**)



To 4-nitrobromobenzene (1g, 4.95mmol), palladium acetate (0.033g, 0.149mmol), triphenylphosphine (0.026, 0.099mmol) and sodium carbonate (1.05g, 9.9mmol) in 20ml NMP was added methyl-methacrylate under N_2. This was then heated to 130°C for 5 hours. After which time, the reaction mixture was cooled to room temperature, filtered through a small pad of celite, diluted with Et_2O (20ml) and washed with 0.1M HCl (20ml) and H_2O (2

x 20ml). The organic layer was then dried over $MgSO_4$ and concentrated. The crude oil was purified by flash column chromatography (100% hexane). The solvent was removed under reduced pressure to yield the title compound as a yellow solid. Yield 0.22g, 22%. Rf = 0.44, 2:8, EtOAc:hexane.

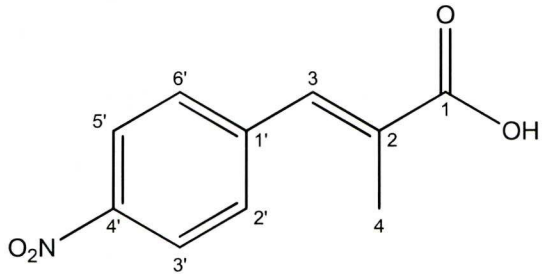
^1H (400MHz, $CDCl_3$) δ: 2.13 (3H, s, H-4), 3.85 (3H,s, H-5), 7.53 (2H, d, J = 8.39, H-2',6'), 7.70 (1H, s, H-3), 8.26 (2H, d, J = 8.88, H-2'6').

^{13}C (100MHz, $CDCl_3$) δ: 14.63 (C-4), 52.82 (C-5), 124.07 (C2',6'), 130.60 (C-3',6'), 132.37 (C-2) 136.70 (C-1'), 142.00 (C-4'), 146.61 (C-3), 169.98 (C-1).

υ_{max} /cm^{-1} 2979.48 (C-H), 1508.06, 1336.43 (NO_2); 1627.63 (C=C), 1702.84 (C=O)

HRMS calcd for $C_{11}H_{11}O_4NNa$ (M+Na) 244.0586; found 244.0597.

5.13.11 (*E*)-2-Methyl-3-(4-nitrophenyl)acrylic acid[186] (**194**)



To compound **193** (45mg, 0.255mmol) in in MeOH (2ml) was added 2ml 5*N* NaOH solution and the mixture allowed to stir at room temperature for 3 days. This was then acidified with the dropwise addition of 1M HCl to pH 2. The product was extracted into ether (2 x 5ml) and washed consecutively with brine (5ml) and water (5ml). The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and chromatographed on silica gel using an eluent system of 50:50 EtOAc: Hexane. The solvent was removed under reduced pressure to yield the title compound as a yellow solid. Yield 0.035g, 83%. Rf = 0.23 1:1 EtOAc:hexane.

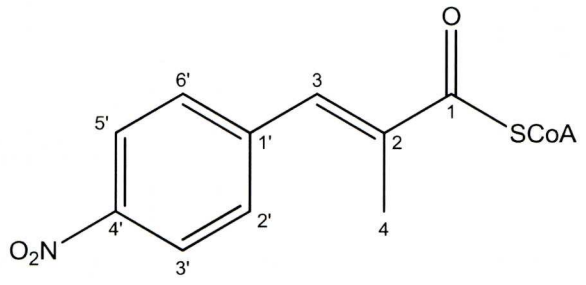
^1H (400MHz, $CDCl_3$) δ: 2.00 (3H, s, H-4), 7.55 (2H, d, J = 8.39, H-2',6'), 7.64 (1H, s, H-3), 8.20 (2H, d, J = 8.88, H-3',5').

^{13}C (100MHz, $CDCl_3$) δ: 14.75 (C-4), 125.00 (C-2',6'), 130.09 (C-2) 132.01 (C-3',5'), 133.94 (C-3) 137.83 (C-1'), 144.37 (C-4'), 171.62 (C-1).

υ_{max} /cm^{-1} 2979.48 (C-H), 1511.92, 1344.14 (NO_2); 1683.55 (C=O).

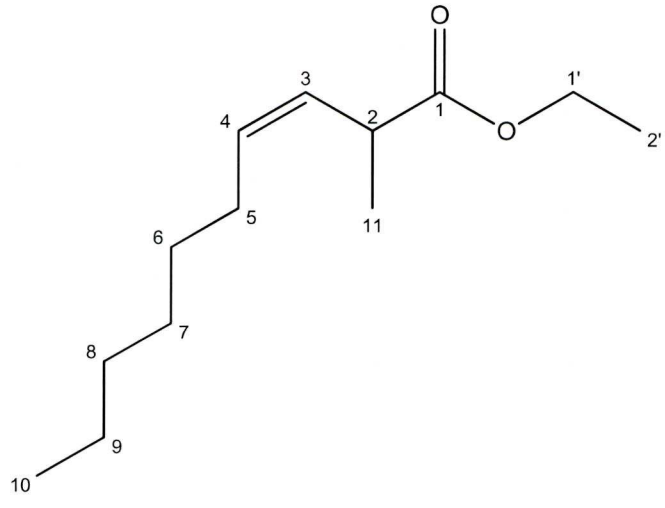
HRMS calcd for $C_{20}H_{17}O_8N_2$ (2M-H^-) 413.0985; found 413.0998.

5.13.12 (*E*)-2-Methyl-3-(4-nitrophenyl)acryloyl-CoA (**71**)



To a solution of compound **194** (20mg, 0.096mmol) in THF (0.5ml) was added NEt_3 (13.4µl, 0.096mmol) followed by ethyl chloroformate (9.2µl, 0.096mmol) at 0°C. This was brought to room temperature and allowed to stir for 4 hours. After which time CoA-SH, $(Li^+)_3$ (7.5mg, predissolved in 0.5ml H_2O) was added and the reaction was allowed to stir over night. The reaction medium was then subject to HPLC purification to afford the title compound as a crystalline solid Yield = 1.38mg, 15% based on relative HPLC traces of coenzyme A and product. HRMS calcd for $C_{31}H_{42}N_8O_{19}P_3S$ 955.1500 ($M+H^-$); found 955.1490.

5.13.13 (Z)-Ethyl 2-methyldec-3-enoate (**200**)



To diisopropylamine (0.7ml, 5mmol) in THF (10ml) was added *n*-BuLi (2ml, 2.5M, 5mmol) at 0°C. This was allowed to stir for 30 mins at that temperature. The reaction is then cooled to -78°C and DMPU (0.6ml, 5mmol) was added. This was allowed to stir for a further 30 mins. After which time (*E*)-ethyl dec-2-enoate (1g, 1.13ml, 5mmol), predissolved in 2ml THF, was added dropwise. This was allowed to stir at 30 minutes at that temperature to which methyl iodide (0.31ml, 4.98mmol) was added dropwise and the reaction mixture was allowed to stir

at that temperature for 2 hours. The reaction mixture was then poured into ice and extracted with diethyl ether (2 x 20ml). This was then washed with Brine (40ml) followed by H_2O (40ml), dried over Na_2SO_4 and evaporated to dryness under reduced pressure. This was then chromatographed on silica gel using 1:9 Et_2O: hexane to yield the titled compound as a colourless oil. Yield 0.48g, 45% Rf = 0.6, 1:9 Et_2O:hexane.

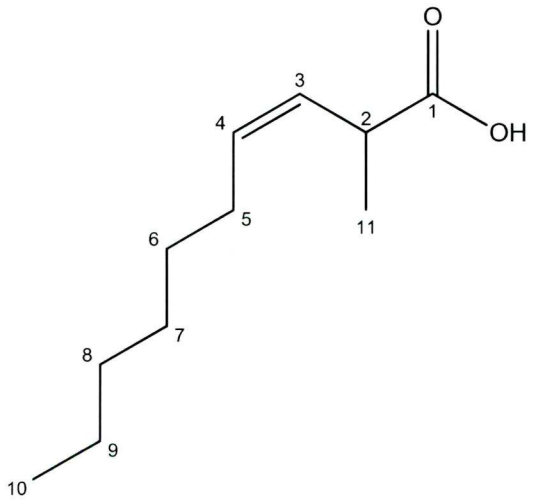
^1H (400MHz, $CDCl_3$) δ: 0.81 (3H, t, *J* = 7.20 Hz, H-10), 1.15 (3H, t, *J* = 7.13 Hz H-2') 1.21 (11H, m, H-6-9, 11) 2.01 (2H, q, *J* = 7.20 Hz, H-5) 3.34 (1H, dq, *J* = 7.00 Hz, 9.2 H-2), 4.05 (2H, q, 7.15 Hz, H-1'), 5.31, (1H, tt *J* = 10.01, 1.42 Hz, H-4), 5.39 (1H, m, H-3).

^{13}C (100MHz, $CDCl_3$) δ: 14.54 (C-2'), 14.62 (C-10), 18.46 (C-11), 23.01-32.79 (C-5-9), 38.50 (C-2), 60.77 (C-1'), 128.99 (C-4) 132.25 (C-3), 175.53 (C-1).

υ_{max} /cm^{-1} 2969.84 (C-H), 1708.62 (C=O) 1616.36 (C=C).

HRMS calcd for $C_{13}H_{25}O_2$ ($M+H^+$) 213.18545; found 213.18574

5.13.14 (*Z*)-2-Methyldec-3-enoic acid (**201**)



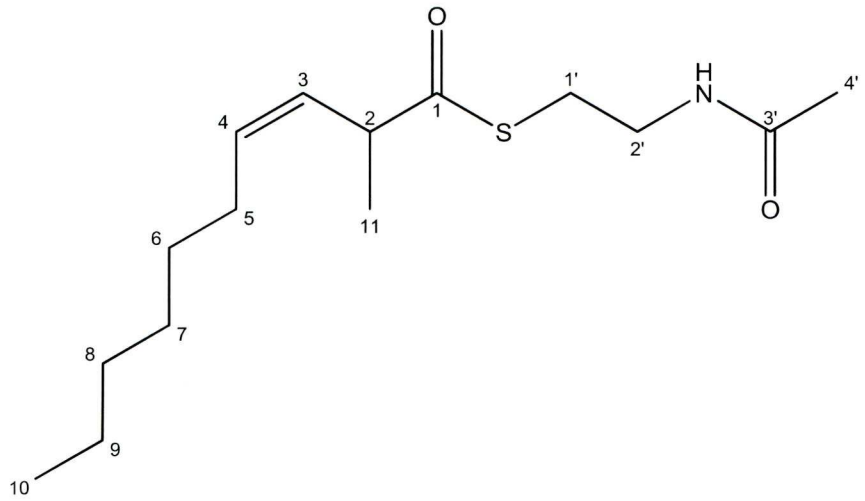
To compound **206** (2.2g, 10.38mmol) in 85ml H_2O was added $Ba(OH)_2.8H_2O$ (6.5g, 20.7mmol). This was allowed to reflux for 4 hours. The precipitated barium salt was filtered from the hot solution and the filtrate was transferred immediately to a cool (25°C) solution of 1M H_2SO_4 (18 ml). This was extracted with Et_2O (2 x 50ml) and washed with H_2O (2 x 50ml). The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and purified by flash column chromatography using an eluent system of 3:7 Et_2O: Hexane + 3% acetic acid to yield the desired compound as a white solid. Yield 1.53g, 80%. Rf = 0.36, 3:7 Et_2O: Hexane + 3% acetic acid.

^{1}H (400MHz, $CDCl_3$) δ: 0.811 (3H, t, *J* = 6.90 Hz, H-10), 1.18 (3H, d, *J* = 7.02 Hz H-11) 1.19-1.34 (8H, m, H-6-9),) 2.01 (2H, dq, *J* = 7.30, 1.30 Hz, H-5) 3.38 (1H, dq, *J* = 7.02, 0.73 Hz H-2), 5.34, (1H, tt, *J* = 10.01, 1.52 Hz, H-4), 5.39 (1H, m, H-3);

^{13}C (100MHz, $CDCl_3$) δ: 14.44 (C-10), 18.29 (C-11), 22.91-32.79 (C-5-9), 38.39 (C-2), 128.40 (C-4) 133.37 (C-3), 182.04 (C-1) ; $_{max}$ /cm^{-1} 3392 (OH), 2969.84 (C-H), 1699.81 (C=O) 1617.15 (C=C);

HRMS calcd for $C_{11}H_{24}O_2N$ ($M+NH_4^+$) 202.1807; found 202.1814.

5.13.15 (*Z*)-S-(2-Acetamidoethyl) 2-methyldec-3-enethioate (**72**)

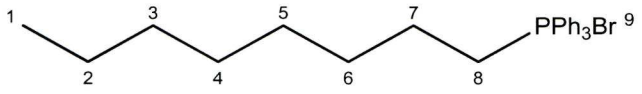


To carbonyldiimidazole (0.13g, 0.81mmol) in THF (5ml) was added compound **207** (0.150g, 0.81mmol) (predissolved in 1ml THF). This was allowed to stir at room temperature for 2 hours. To this was then added *N*-acetylcysteamine (0.086ml, 0.81mmol) dropwise and the reaction mixture was then allowed to stir over night. The solvent was evaporated and the residue was re-dissolved in Et_2O (15ml). This was washed with H_2O (2*20ml), dried over $MgSO_4$, and concentrated *in vacuo*. The colourless oil was purified using column chromatography using an eluent of 1:9 MeOH:DCM and dried under reduced pressure to yield the title compound as a colourless oil. Yield = 0.148g, 64%. Rf = 0.43, 1:9 MeOH:DCM.

^{1}H (400MHz, $CDCl_3$) δ: 0.89 (3H, t, *J* = 6.88 Hz, H-10), 1.25 (3H, d, *J* = 6.92 Hz, H-11) 1.37-1.42 (6H, m, H-6-9),) 1.95 (3H, s, H-4') 2.09 (2H, m, H-5) 3.00 (2H, t, *J* = 6.50 Hz, H-1'), 3.42 (2H, q, *J* = 6.2, H-2') 3.48 (1H, q, *J* = 7.02, H-2) 5.35 (1H, dd, 11.51, 10.69 Hz, H-3), 5.57 (1H, dt *J* = 10.69, 7.35 Hz, H-4) 5.85 (1H, s, NH);

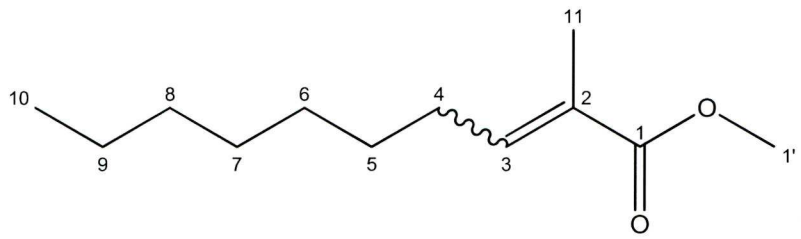
^{13}C (100MHz, $CDCl_3$) δ: 14.49 (C-10), 18.54 (C-11), 23.03-34.36 (C-5-9, 1',4'), 40.14 (C-2'), 47.44 (C-2), 128.16 (C-3) 134.11 (C-4), 170.62 (C-3'), 202.83 (C-1);
HRMS calcd for $C_{15}H_{27}O_2NS$ ($M+H^+$) 286.18408; found 286.18390.

5.13.16 Bromo(octyl)triphenylphosphorane (**204**)



Compound **210** was prepared in an identical manner as described in Section 5.11.1, using triphenylphosphine (2.72g, 10.35mmol) and 1-bromo-octane (2g, 1.79ml, 10.35mmol) in dry toluene (20ml) to yield the title compound as a white solid. Yield = 4.6g, 99%.
^1H NMR (400 MHz, $CDCl_3$) δ: 0.75 (3H, t, J = 6.96 Hz, H-1), 1.15-1.36 (22H, m, H-2-7), 1.40-1.45 (2H, m, H8) 7.82-7.11 (15H, m, H-9). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.44 (C-1), 22.99 (C-2), 29.10-34.51 (C-3-8), 119.16-135.46 (18C, C-9), (Cl) m/z for $C_{26}H_{32}P$ (M+): 375 (100.00).

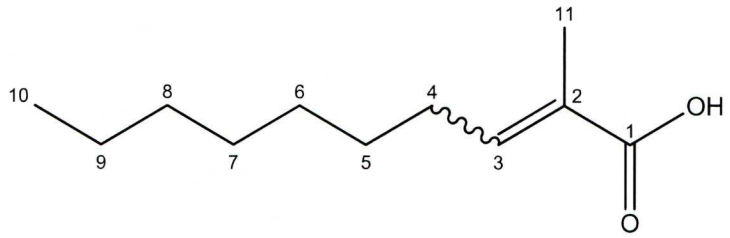
5.13.17 (*E/Z*)-Methyl 2-methyldec-2-enoate[187] (**205**)



Compound **210** (4.55g, 10mmol) was subjected to the same conditions as described in Section 5.11.2, using THF (75ml), 2.5M *n*-BuLi (4.42ml, 11mmol) and methyl pyruvate (1.02g, 0.9ml, 10mmol). The titled compound was purified by flash column chromatography using 10:90 Et_2O:hexane to yield the title compound as a colourless oil. Yield = 0.89g, 45%.
Rf (*Z*) = 0.62, (*E*) = 0.70, 2:8 Et_2O:hexane.
(*Z*)-isomer δ ^1H (400MHz, $CDCl_3$) δ: 0.81 (3H, t, J = 3.37 Hz, H-1), 1.22 (10H, m, H-2-6) 1.30-1.37 (2H, m, H7) 1.76 (3H, s, H-12) 3.67 (3H, s, H-11) 5.97 (1H, dt, J = 7.4, 1.52 Hz, H-8); ^{13}C (100MHz, $CDCl_3$) 12.74 (C-1), 14.47-32.21 (C-2-7, 12), 51.56 (C-11), 127.02 (C-9), 143.26 (C-8) 168.97 (C-10).

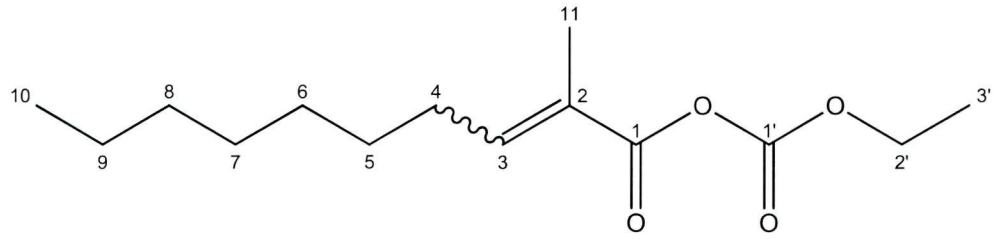
(*E*)-isomer ^{1}H (400MHz, $CDCl_3$) δ: 0.81 (3H, t, *J* = 3.37 Hz, H-10), 1.22 (10H, m, H-5-9) 1.30-1.37 (2H, m, H-4) 1.82 (3H, s, H-1') 3.67 (3H, s, H-11) 6.99 (1H, dt, *J* = 7.44, 1.42 Hz, H-3); ^{13}C (100MHz, $CDCl_3$) 12.74 (C-10), 14.47-32.21 (C-4-9,1'), 52.04 (C-11), 127.75 (C-2), 144.22 (C-3) 169.19 (C-1); $_{max}$ /cm^{-1} 2979.48 (C-H), 1716.34 (C=O), 1645.45 (C=C);
HRMS calcd for $C_{12}H_{23}O_2N$ ($M+H^+$) 199.16980; found 199.16980

5.13.18 (*E/Z*)-2-Methyldec-2-enoic acid[188] (**206**)



Compound **212** was synthesised in an analogous manner to compound (**188**), using (*E/Z*)-methyl 2-methyldec-2-enoate (**211**) (0.5g, 2.52mmol), methanol (5ml), and 5*N* NaOH (2ml). This was then purified by flash column chromatography using 20:80 Et_2O:hexane to yield the title compound as a colourless oil (0.33g, 70%) . Rf (*E/Z*) = 0.17, 2:8 Et_2O:hexane;
(*E*)-isomer ^{1}H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 6.93 Hz, H-10), 1.22-1.49 (10H, m, H-5-9), 1.92 (3H, s, H-11), 2.51 (2H, dq, *J* = 7.4, 1.33 H-4) 6.95 (1H, dt, *J* = 7.55, 1.422 Hz, H-3);
^{13}C (100MHz, $CDCl_3$) δ: 12.34 (C-11), 20.87 (C-10) 28.84-32.21 (C-4-9), 126.46 (C-2), 145.97 (C-3) 174.31 (C-1).
(*Z*)-isomer ^{1}H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 6.93 Hz, H-10), 1.22-1.49 (10H, m, H-5-9) 1.82 (3H, s, H-11), 2.20 (2H, dq, *J* = 7.4, 0.66 Hz, H-4) 6.10 (1H, dt, *J* = 7.44, 1.43 Hz, H-3);
^{13}C (100MHz, $CDCl_3$) δ: 14.48 (C-11), 23.06 (C-10) 28.84-32.21 (C-4-9), 127.33 (C-2), 147.45 (C-3) 174.31 (C-1). υ_{max} /cm^{-1} 3400 (C-OH); 1699.84 (C=O), 1645.45 (C=C);
HRMS calcd for $C_{11}H_{24}O_2N$ ($M+NH_4^+$) 202.18070; found 202.18140

5.13.19 2-Methyldec-2-enoic propionic anhydride (**215**)



To a solution of compound **212** (0.078g, 0.42mmol) in THF (2ml) was added NEt_3 (0.058ml, 0.42mmol) followed by ethyl chloroformate (0.04ml, 0.42mmol). This was allowed to stir at room temperature for 4 hours. After which time the solvent was removed followed by introduction of Et_2O (5ml). This was subsequently washed with H_2O (2*5ml) followed by brine (5ml). The organic layer was dried over Na_2SO_4 and purified by flash column chromatography to yield the title compound as a colourless oil. Yield: 0.082g, 82% Rf = 0.41, 2:8 Et_2O:hexane;

(*E*)-isomer ^1H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 6.94 Hz, H-10), 1.25-1.47 (10H, m, H-5-9), 1.38 (3H, t, *J* = 7.15 Hz, H-3'), 1.92 (3H, s, H-11), 2.51 (2H, dq, *J* = 7.51, 1.33 H-4), 4.34 (2H, q, J = 7.14 Hz, H-2'), 6.95 (1H, dt, *J* = 7.51, 1.36 Hz, H-3);

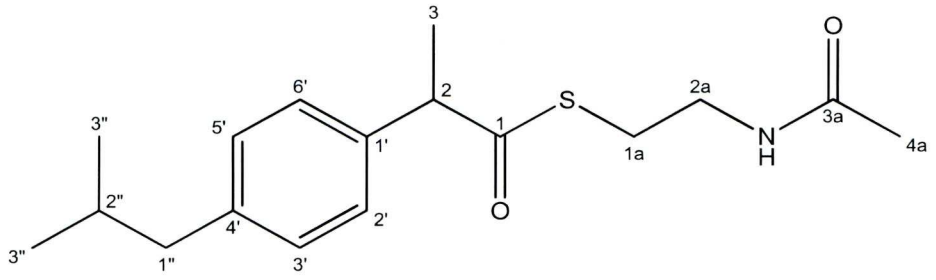
^{13}C (100MHz, $CDCl_3$) δ: 12.59 (C-11), 20.66 (C-10) 28.67-32.18 (C-4-9), 65.99 (C-2') 126.36 (C-2), 148.57 (C-3), 150.22 (C-1') 162.84 (C-1).

(*Z*)-isomer ^1H (400MHz, $CDCl_3$) δ: 0.88 (3H, t, *J* = 6.94 Hz, H-10), 1.25-1.47 (10H, m, H-5-9), 1.38 (3H, t, *J* = 7.15 Hz, H-3'), 1.87 (3H, s, H-11), 2.22 (2H, dq, *J* = 7.42, 0.78 Hz, H-4), 4.34 (2H, q, J = 7.14 Hz, H-2') 6.10 (1H, dt, *J* = 7.45, 1.45 Hz, H-3);

^{13}C (100MHz, $CDCl_3$) δ: 12.59 (C-3'), 14.48 (C-11), 23.06 (C-10) 28.84-32.21 (C-4-9), 65.93 (C-2') 124.91 (C-2), 148.57 (C-3), 150.01 (C-1') 162.07 (C-1).

HRMS calcd for $C_{14}H_{28}O_4N$ ($M+NH_4^+$) 274.20183; found 274.20242

5.13.20 S-(2-acetamidoethyl) 2-(4-isobutylphenyl)propanethioate (**55**)

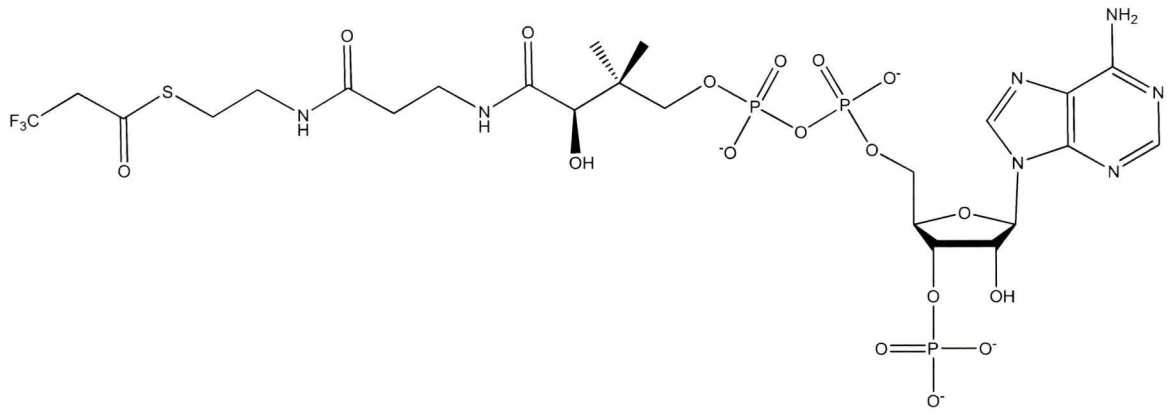


To Ibuprofen (**173**) (0.89 g, 4.3 mmol) in THF (15ml) was added dicyclohexylcarbodiimide (0.89 g, 4.3 mmol) and 1-hydroxybenzotriazole (0.59 g, 4 mmol) followed by *N*-acetylcysteamine (0.52 g, 4.3 mmol). After stirring at room temperature for 45 minutes K_2CO_3 (0.299 g, 2.1 mmol.) was added and the reaction stirred for 3 hours at room temperature. The reaction was then filtered and concentrated *in vacuo*. The residue was taken up in ethyl acetate (20ml) and washed once with 10% aqueous $NaHCO_3$ (20ml). The organic layer was dried over Na_2SO_4 and concentrated. The crude product was purified by flash chromatography (3–5% MeOH in CH_2Cl_2) and concentrated. Yield 0.77g, 59%. Rf = 0.47 1:9 MeOH:DCM

^{1}H NMR (400MHz, $CDCl_3$) δ: 0.83 (6H, d, *J* = 6.81 Hz H-3"), 1.50 (3H, d, *J* = 7.1 Hz, H-3), 1.88-1.80 (4H, m, H-2",4a) 2.44 (2H, d, J = 7.18 Hz, H-1"), 3.36 (2H, *J* = 6.1 Hz, H-1a), 3.68 (1H, q, *J* = 7.09 Hz, H-2), 3.88 (2H, m, H-2a), 7.18 (2H, d, *J* = 6.33 Hz, H-3',5'), 7.28 (2H, d, J = 6.33 Hz, H-2',6').

^{13}C NMR (100MHz, $CDCl_3$) δ: 18.57 (C-3). 22.78 (C-3"), 23.27 (C-4a), 30.34 (C-2"), 30.53 (C-1a), 40.01 (C-2a), 45.45 (C-1"), 54.44 (C-2), 126.88-141.52 (C-1',2',3',5',4',6'), 171.23 (C-3a), 204.11 (C-1), (Cl) m/z for $C_{17}H_{29}NO_2S$ 325 ($M + NH_4^+$): 137 (100.00)

5.13.21 Trifluoropropionyl-CoA (**211**)



To trifluoropropionic acid (0.021mg, 0.23mmol) was added carbonyldiimidazole (0.038g, 0.23mmol) in THF (1ml). This was allowed to stir at room temperature for 2 hours. After which time the solvent is removed under reduced pressure and the oil/solid mixture was re-dissolved in Et_2O (2ml) and washed three times with H_2O (3*2ml). The organic layer was dried over Na_2SO_4 and removed under reduced pressure. The colourless oil was resuspended in THF (0.5ml) and to this was added CoA-SH, $(Li^+)_3$ (20mg, 25.51μmol, predissolved in 0.5ml H_2O). This was allowed to stir at room temperature over night. The reaction mixture was then subject to HPLC purification to yield the title compound (**216**) 4.03mg, 18% yield-based on relative HPLC traces of coenzyme A and product.

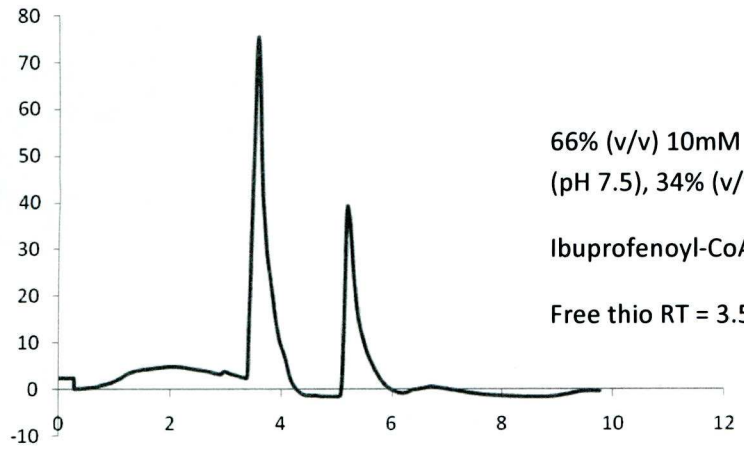
^1H (400MHz, $CDCl_3$) δ: 0.69, (3H, s, H-7/8), 0.82 (3H, s, H-7/8), 2.35 (2H, t, 6.52 Hz, H-4), 2.99 (2H, m, H-2), 3.07 (2H, q, J = 11.38hz, H-1), 3.28 (4H, m, H-5), 3.37 (2H, m, H-3) 3.50 (2H, m, H-19), 3.77 (2H, m, H-10), 3.99 (1H, s, H-6), 4.18 (2H, m, H-17-18), 4.52 (1H, m, H-19), 6.06 (1H, d, *J* = 6.56Hz , H-20), 8.12 (1H, s, H-21), 8.45 (1H, s, H-22)

HRMS calcd for $C_{24}H_{36}F_3N_7O_{17}P_3S$ ($M+H^-$) 876.1065; found 876.1053

APPENDICES

Appendix 1

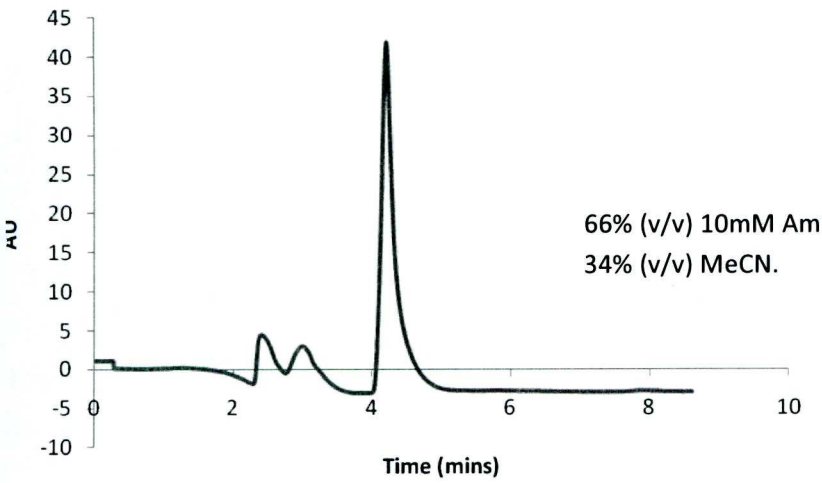
HPLC traces of Ibuprofenoyl-CoA at various pH's cont



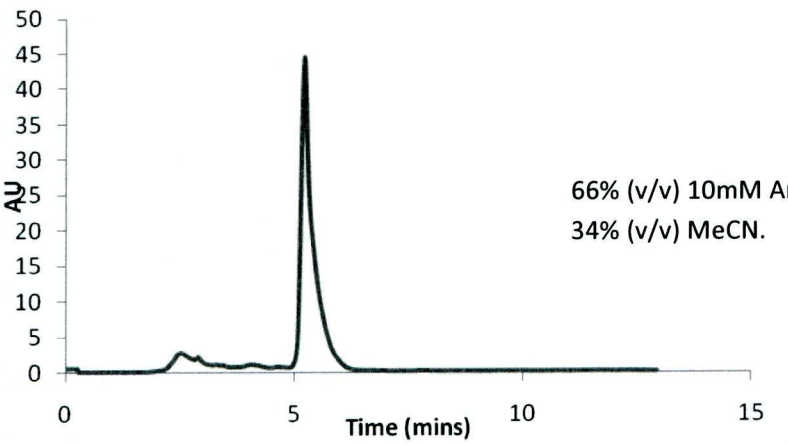
66% (v/v) 10mM Ammonium acetate-ammonium hydroxide, (pH 7.5), 34% (v/v) MeCN.

Ibuprofenoyl-CoA RT = 5.21

Free thio RT = 3.59 **significant hydrolysis**

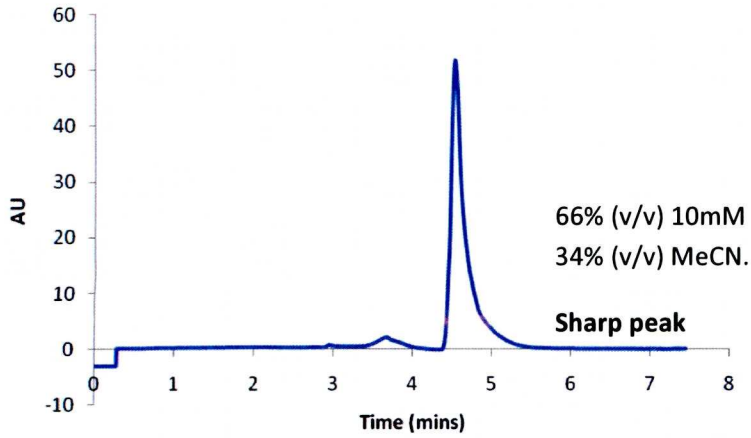


66% (v/v) 10mM Ammonium acetate-acetic acid, (pH 7), 34% (v/v) MeCN.



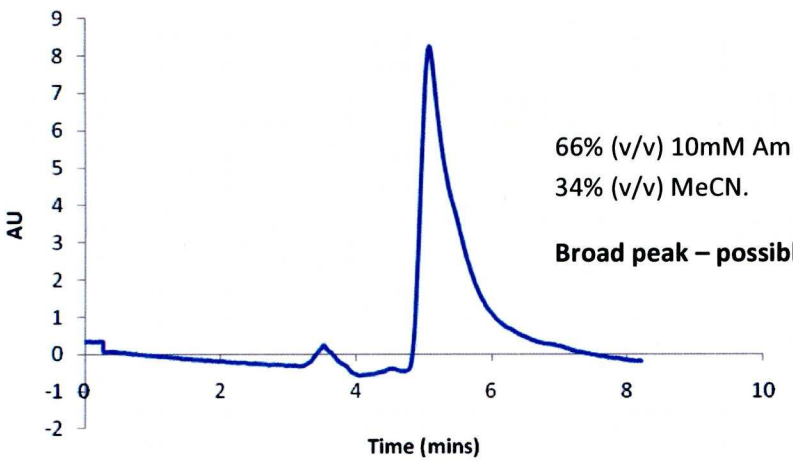
66% (v/v) 10mM Ammonium acetate-acetic acid, (pH 6.5), 34% (v/v) MeCN.

HPLC traces of Ibuprofenoyl-CoA at various pH's cont



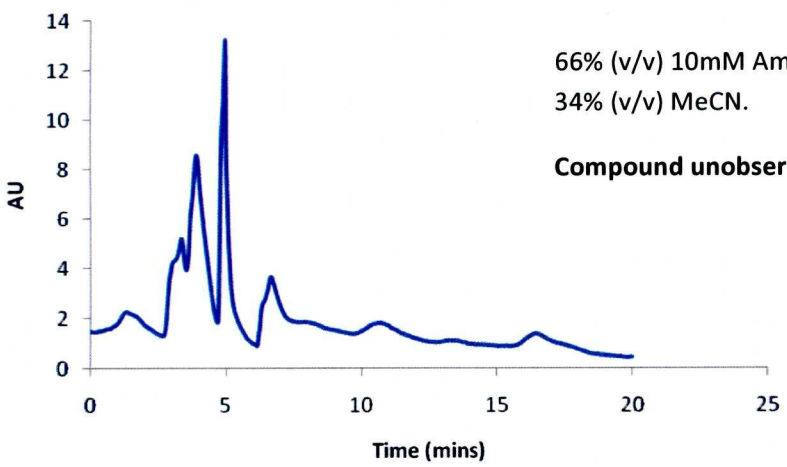
66% (v/v) 10mM Ammonium acetate-acetic acid, (pH 6),
34% (v/v) MeCN.

Sharp peak



66% (v/v) 10mM Ammonium acetate-acetic acid, (pH 5.5),
34% (v/v) MeCN.

Broad peak – possibly due to multiple protonation states.



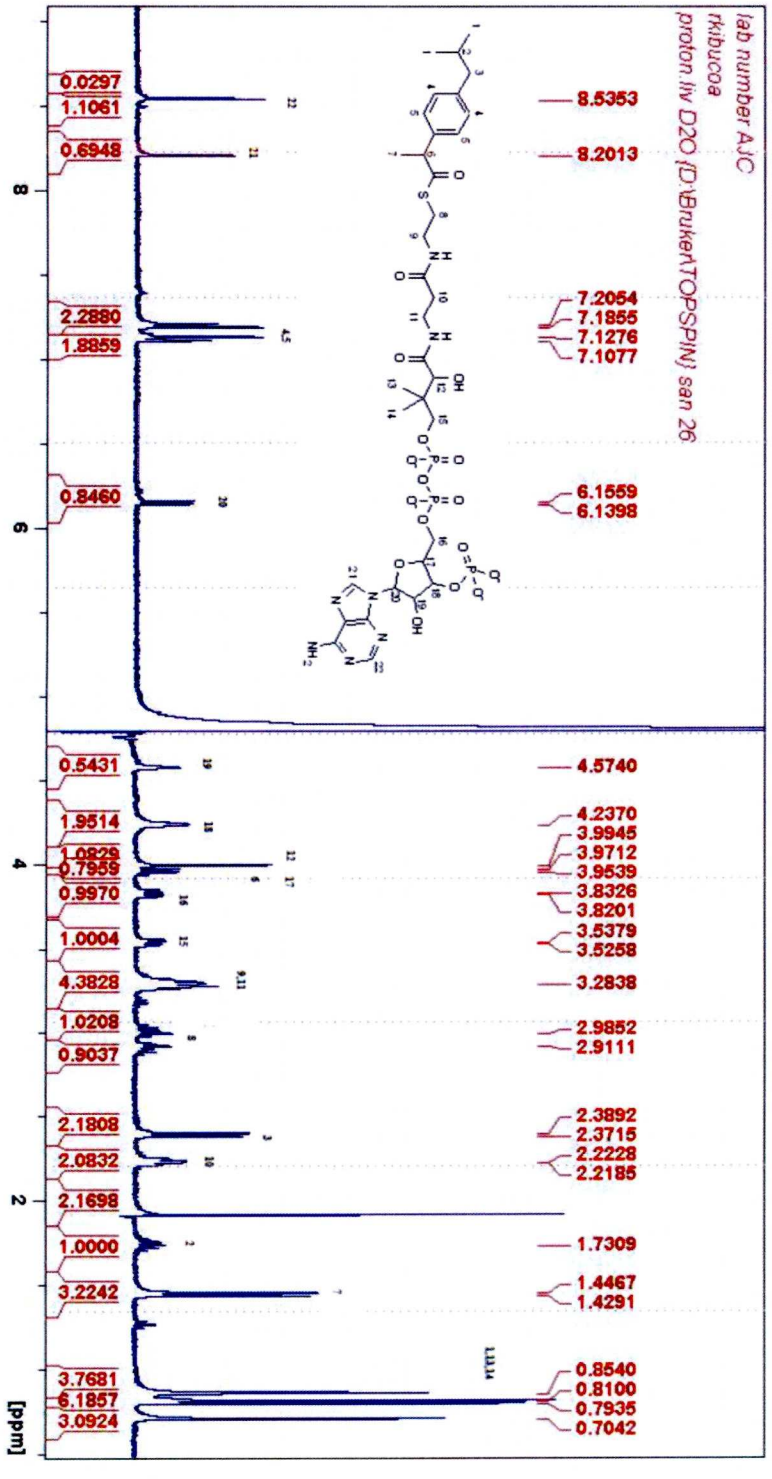
66% (v/v) 10mM Ammonium acetate-acetic acid, (pH 3.5),
34% (v/v) MeCN.

Compound unobservable.

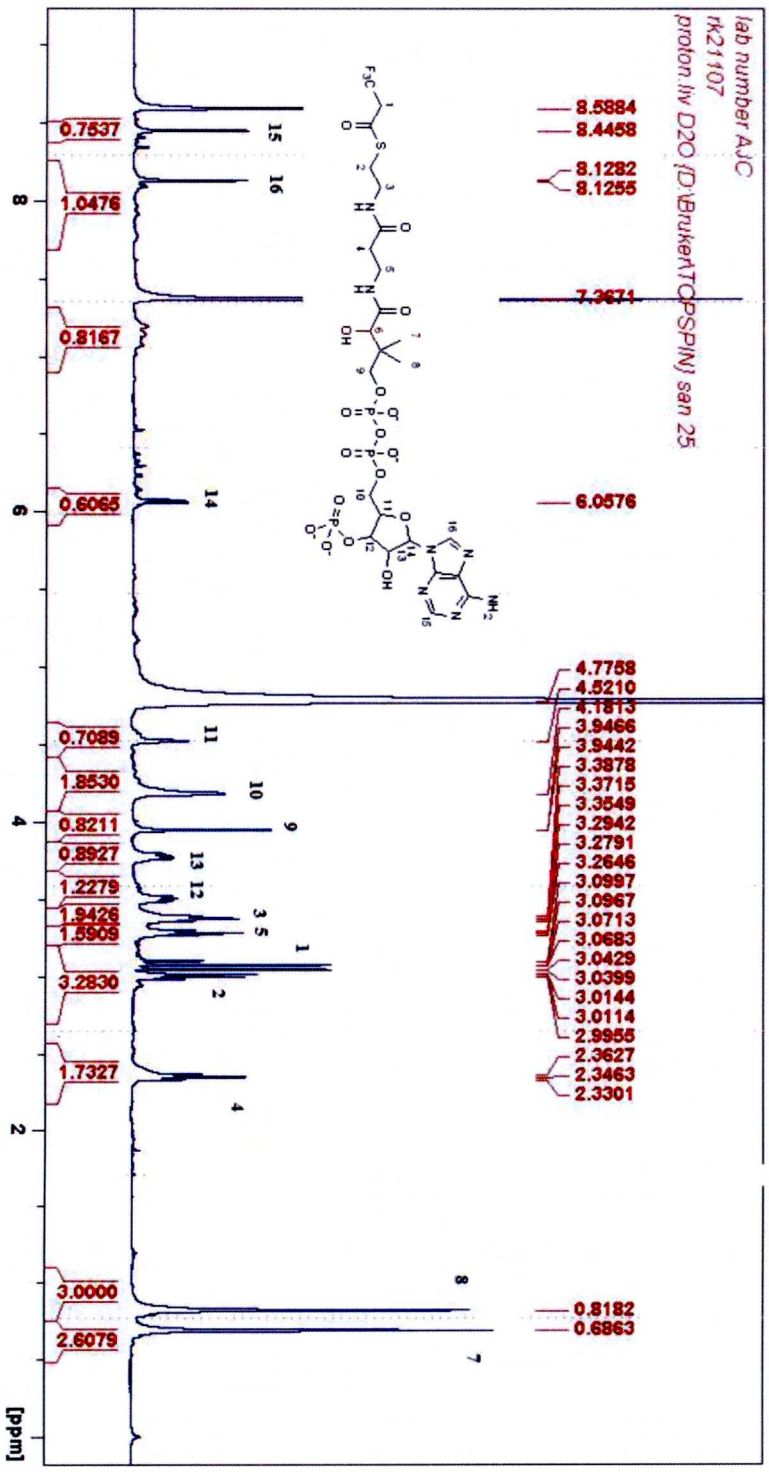
Appendix 2

NMR's of CoA coupled substrates

Ibuprofenoyl-CoA

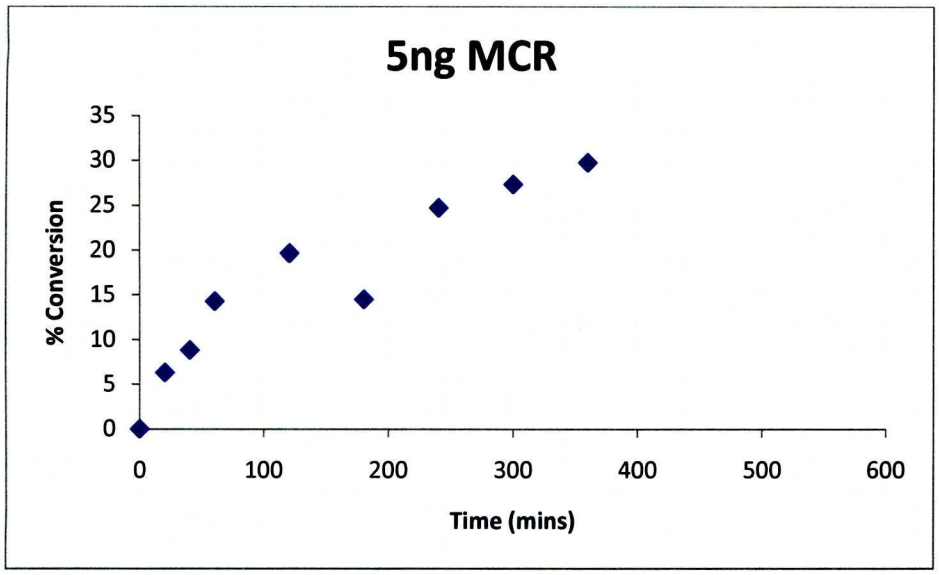


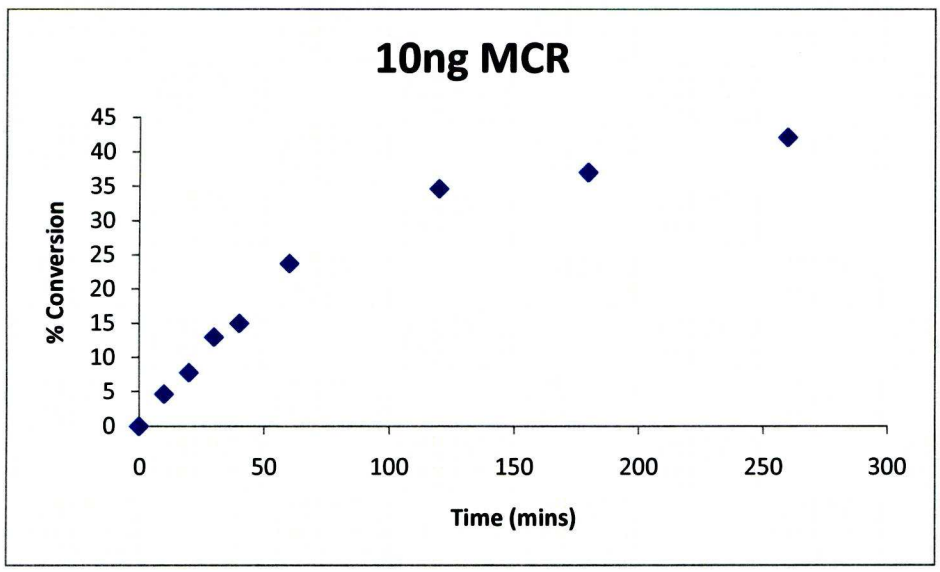
Trifluoropropionyl-CoA

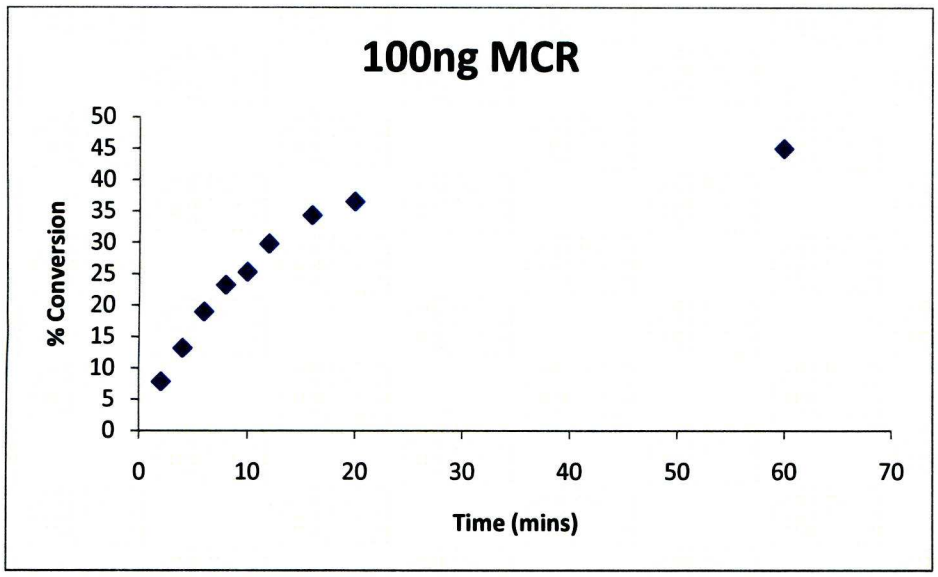


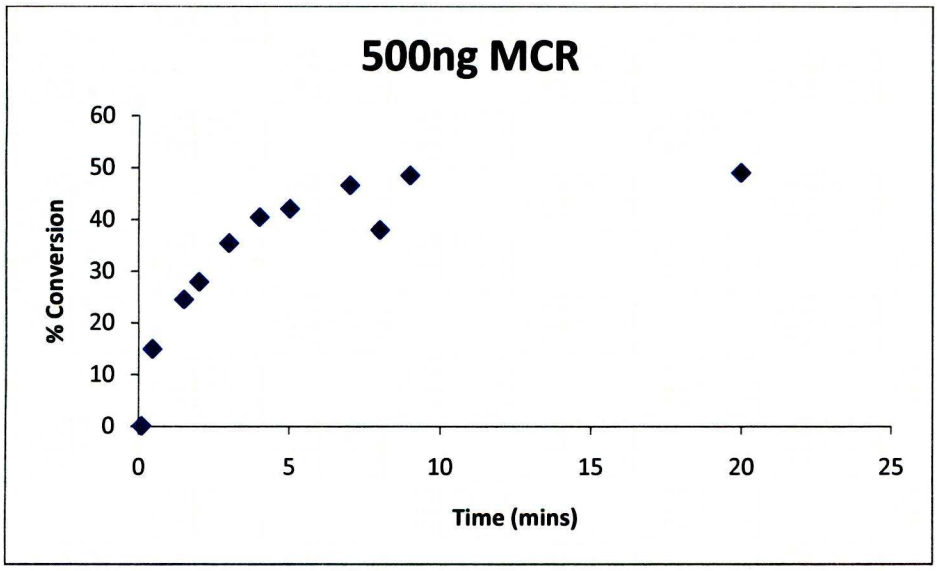
Appendix 3.

Incubations of 100μM (*S*)-Ibuprofenoyl-CoA at various protein concentrations at 37°C and at pH 7.






100ng MCR
% Conversion
50
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Time (mins)


500ng MCR
% Conversion
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (mins)

Appendix 3 continued

Raw data for substrate concentration variation

200μM (*S*)-Ibuprofenoyl-CoA

Time	(%R)	μmoles converted
0	0	0
4	4.05	0.0081
6	6.47	0.01294
8	9.92	0.01984
12	13.56	0.02712
16	13.99	0.02798
18	16.95	0.0339
40	26.19	0.05238
120	38.16	0.07632

100μM (*S*)-Ibuprofenoyl-CoA

Time	(%R)	μmoles converted
0	0	0
6	17.83	0.01783
10	25.2	0.0252
14	29.67	0.02967
20	31.46	0.03146
60	41.26	0.04126

50μM (*S*)-Ibuprofenoyl-CoA

Time	(%R)	μmoles converted
0	0	0
2	8.2	0.0041
8	17.66	0.00883
12	21.07	0.010535
20	25.36	0.01268
46	35	0.0175

Appendix 4

Raw data for assay comparisons

Mass Spec assay of 100ng MCR with 100µM [D]-Ibu-CoA

Time	Area of [H]-IbuCoA	Area of [D]-IbuCoA	% conversion	µmoles converted
0	0	0	0	0
2	2.02	10.72	15.855573	0.015855573
4	3.12	7.87	28.38944495	0.028389445
6	2.99	4.85	38.1377551	0.038137755
8	4.4	5.077	46.42819458	0.046428195
10	4.77	4.08	53.89830508	0.053898305
12	5.17	3.68	58.4180791	0.058418079
14	5.69	3.65	60.92077088	0.060920771
16	5.99	3.29	64.54741379	0.064547414
18	6.74	3.26	67.4	0.0674
20	6.1	2.67	69.55530217	0.069555302
30	7.11	2.15	76.78185745	0.076781857
40	7.83	1.88	80.63851699	0.080638517
60	8.56	2.02	80.9073724	0.080907372

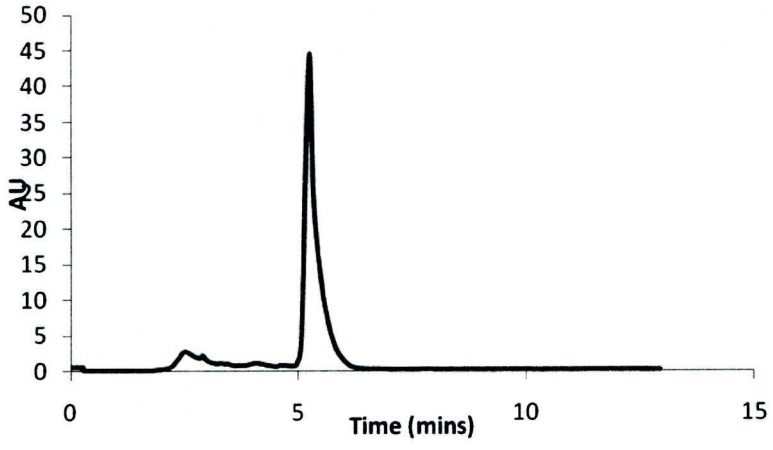
HPLC assay of 100ng MCR with 100µM (*S*)-Ibu-CoA

time	% (R)	% (S)	µmoles converted
0	0	100	0
2	9.73	90.27	0.00973
4	14	86	0.014
6	22.09	77.91	0.02209
8	22.12	77.88	0.02212
10	24.5	75.5	0.0245
12	31.49	68.51	0.03149
16	30.73	69.27	0.03073
18	35.43	64.57	0.03543
20	34.59	65.41	0.03459
60	43.06	56.94	0.04306

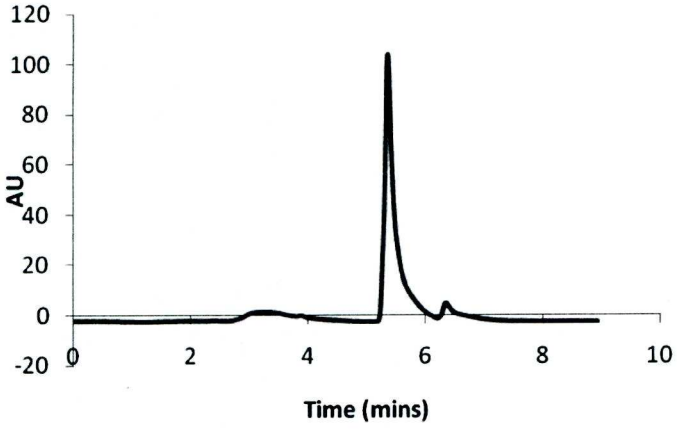
Appendix 5

HPLC traces of various CoA thioesters

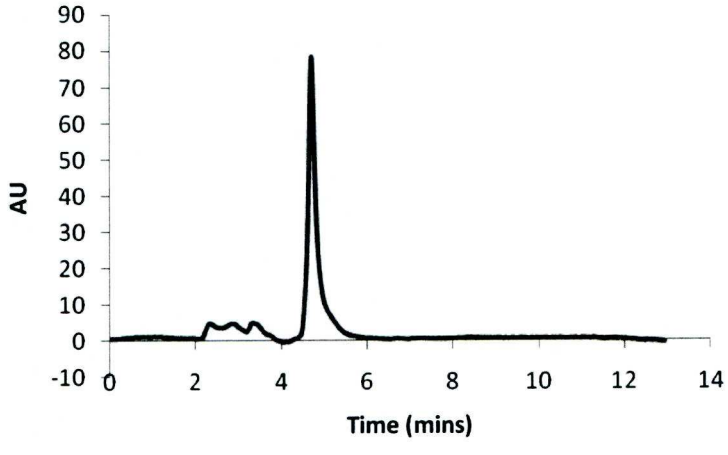
Ibuprofenoyl-CoA



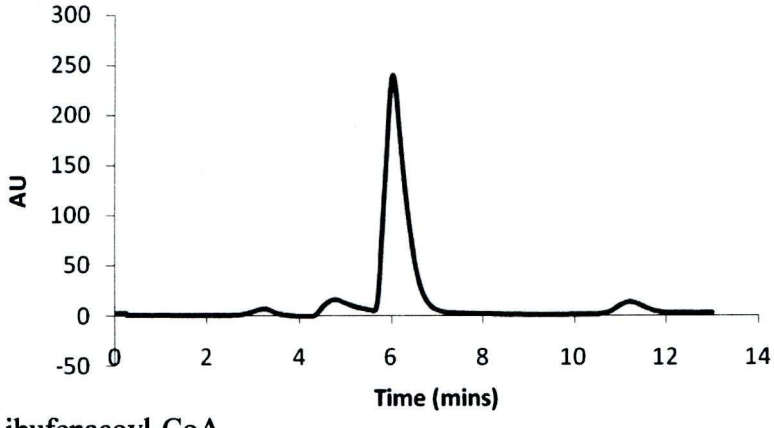
α-deutero-ibuprofenoyl-CoA



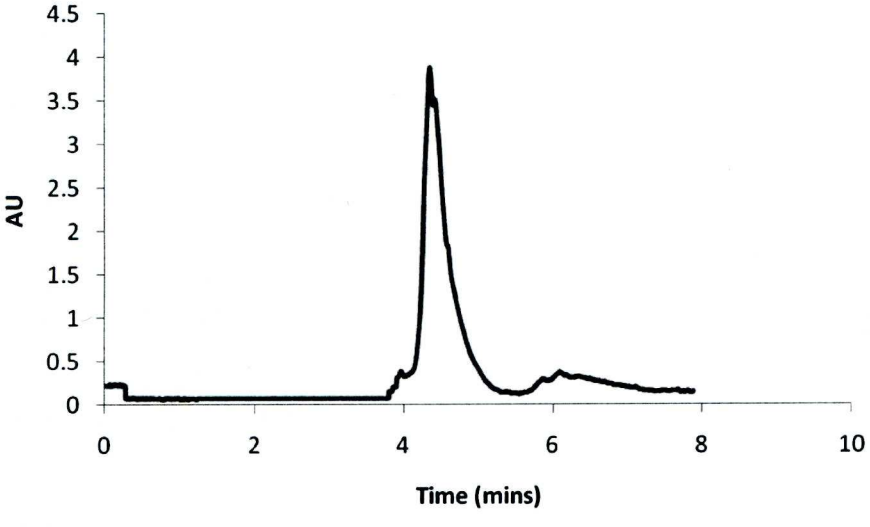
α-fluoro-ibuprofenoyl-CoA



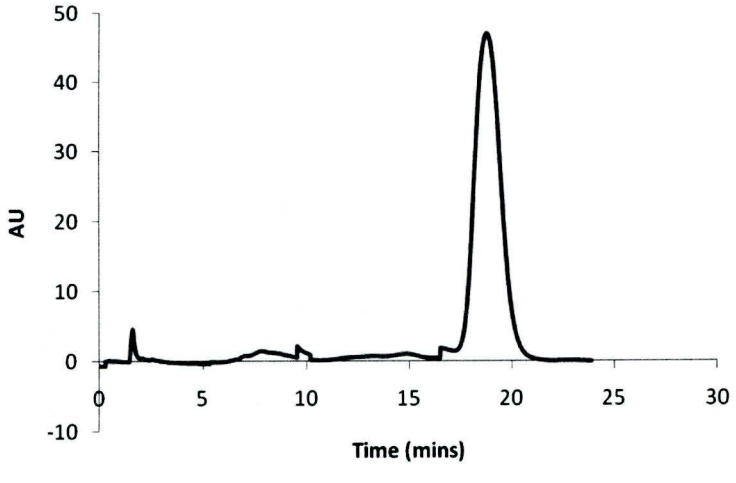
Ibufenacoyl-CoA



α-chloro-ibufenacoyl-CoA



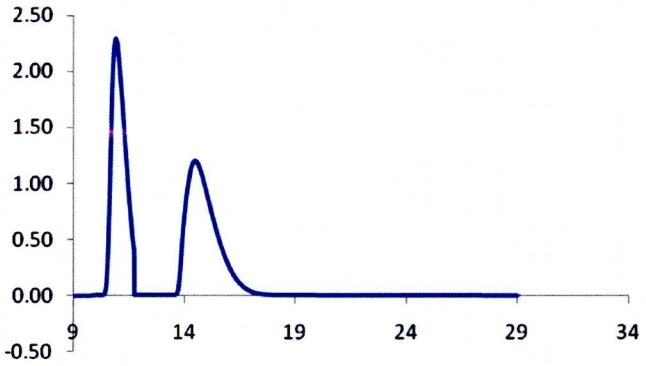
2-Trifluorotetradecanoyl-CoA



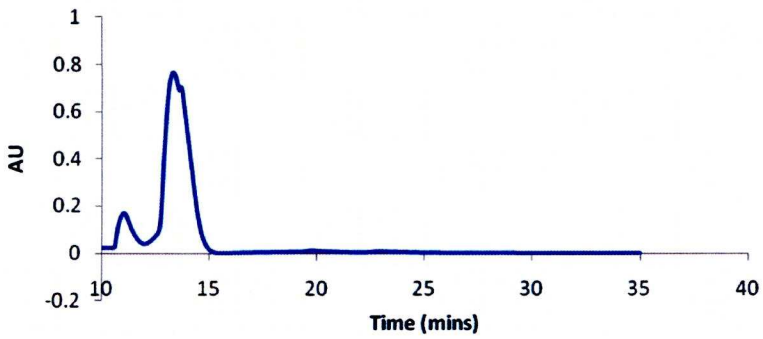
Appendix 6

Enzyme hydrolysis of α–chloroibufenac ethyl ester

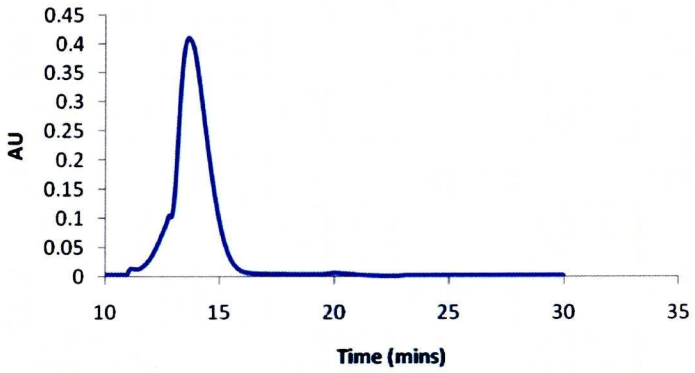
Racemic α–chloroibufenac ethyl ester before incubation



Enzyme Reaction after 3 hours



Enzyme Reaction after 5 hours



REFERENCES

1. S. Zha, S. Ferdinandusse, S. Denis, R. Wanders, C. Ewing, J. Luo, A. De Marzo and W. Isaacs, *Cancer Research*, 2003, **63**, 7365-7376.
2. http://www.who.int/mediacentre/factsheets/fs297/en/, *Cancer - Key Facts*, Accessed 04/05/2010.
3. http://web.archive.org/web/20060205235509/http://www.jncicancerspectrum.oxfordjournals.org/cgi/statContent/cspectfstat;99, cancer statistics, Accessed 04/05/2010.
4. http://info.cancerresearchuk.org/cancerstats/types/prostate/index.htm, *Prostate cancer statistics - Key Facts*, Accessed 04/05/2010.
5. J. Adams, *Lancet*, 1853, **1**, 393.
6. H. Young, *Johns Hopkins Bull*, 1905, **16**, 315.
7. C. Huggins. *Cancer Research.* 1941, **1**, 203.
8. D. Miller, K. Hafez, A. Stewart, J. Montie and J. Wei, *Cancer*, 2003, **98**, 1169-1178.
9. I. van der Cruijsen-Koeter, A. Vis, M. Roobol, M. Wildhagen, H. de Koning, T. van der Kwast and F. Schroder, *Journal of Urology*, 2005, **174**, 121.
10. H. Lilja, *Journal of Clinical Investigation*, 1985, **76**, 1899-1903.
11. http://www.prostate.com/, *Prostate.com,* Accessed 04/05/2010
12. J. Herschman, D. Smith and W. Catalona, *Urology*, 1997, **50**, 239-243.
13. R. Nadler, P. Humphrey, D. Smith, W. Catalona and T. Ratliff, *Journal of Urology*, 1995, **154**, 407-413.
14. L. Banez, R. Hamilton, A. Partin, R. Vollmer, L. Sun, C. Rodriguez, Y. Wang, M. Terris, W. Aronson, J. Presti, C. Kane, C. Amling, J. Moul and S. Freedland, *Journal of the American Medical Association*, 2007, **298**, 2275-2280.
15. E. Crawford, M. Schutz, S. Clejan, J. Drago, M. Resnick, G. Chodak, L. Gomella, M. Austenfeld, N. Stone, B. Miles and R. Thomson, *Journal of the American Medical Association*, 1992, **267**, 2227-2228.
16. T. Fuchikami, Y. Shibata and Y. Suzuki, *Tetrahedron Letters*, 1986, **27**, 3173-3176.
17. Y. Man, C. Zhao and X. Chen, *Pathology - Research and Practice*, 2006, **202**, 651-662.
18. A. Yang, M. Kaghad, Y. Wang, E. Gillett, M. Fleming, V. Dötsch, N. Andrews, D. Caput and F. McKeon, *Neuron* 1998, **2**, 305-316.
19. S. Signoretti, D. Waltregny, J. Dilks, B. Isaac, D. Lin, L. Garraway, A. Yang, R. Montironi, F. McKeon and M. Loda, *American Journal of Pathology*, 2000, **157**, 1769-1775.
20. G. Paner, D. Luthringer and M. Amin, *Archives of Pathology & Laboratory Medicine*, 2008, **132**, 1388-1396.
21. C. Boran, E. Kandirali, F. Yilmaz, E. Serin and M. Akyol, *Urologic Oncology: Seminars and Original Investigations*, doi:10.1016/j.urolonc.2009.11.013
22. V. Tischler, F. Fritzsche, J. Gerhardt, C. Jager, C. Stephan, K. Jung, M. Dietel, H. Moch and G. Kristiansen, *Histopathology*, **56**, 811-815.
23. J. Glenney, *Journal of Biological Chemistry*, 1986, **261**, 7247-7252.
24. J. Glenney, *Proceedings of the National Academy of Sciences of the United States of America*, 1986, **83**, 4258-4262.
25. D. Lee, N. Jamgotchian, S. Allen, F. Kan and I. Hale, *Am J Physiol Renal Physiol*, 2004, **287**, F481-491.
26. A. Smitherman, J. Mohler, S. Maygarden and D. Ornstein, *Journal of Urology*, 2004, **171**, 916-920.
27. D. Yee, N. Narula, I. Ramzy, J. Boker, T. Ahlering, D. Skarecky and D. Ornstein, *Archives of Pathology & Laboratory Medicine*, 2007, **131**, 902-908.
28. J. Luo, S. Zha, W. R. Gage, T. A. Dunn, J. L. Hicks, C. J. Bennett, C. N. Ewing, E. A. Platz, S. Ferdinandusse, R. J. Wanders, J. M. Trent, W. B. Isaacs and A. M. De Marzo, *Cancer Research*, 2002, **62**, 2220-2226.
29. A. Y. Aksinenko, A. N. Pushin and V. B. Sokolov, *Russian Chemical Bulletin*, 2002, **51**, 2136-2138.

30. V. Ananthanarayanan, R. J. Deaton, X. M. J. Yang, M. R. Pins and P. H. Gann, *Prostate*, 2005, **63**, 341-346.
31. A. Gologan, S. Bastacky, T. McHale, J. Yu, C. Cai, F. Monzon-Bordonaba and R. Dhir, *American Journal of Surgical Pathology*, 2005, **29**, 1435-1441.
32. Z. Jiang, B. A. Woda, C. L. Wu and X. M. J. Yang, *American Journal of Clinical Pathology*, 2004, **122**, 275-289.
33. M. A. Rubin, M. P. Zerkowski, R. L. Camp, R. Kuefer, M. D. Hoter, A. M. Chinnaiyan and D. L. Rimm, *American Journal of Pathology*, 2004, **164**, 831-840.
34. P. Teillac and P. Mongiat-Artus, *European Urology Supplements*, 2007, **6**, 728.
35. M. A. Rubin, T. A. Bismar, O. Andren, L. Mucci, R. Kim, R. L. Shen, D. Ghosh, J. T. Wei, A. M. Chinnaiyan, H. O. Adami, P. W. Kantoff and J. E. Johansson, *Cancer Epidemiology Biomarkers & Prevention*, 2005, **14**, 1424-1432.
36. A. J. Evans, *Journal of Clinical Pathology*, 2003, **56**, 892-897.
37. J. I. Epstein, *Modern Pathology*, 2004, **17**, 307-315.
38. C. Magi-Galluzzi, J. Luo, W. B. Isaacs, J. L. Hicks, A. M. De Marzo and J. I. Epstein, *American Journal of Surgical Pathology*, 2003, **27**, 1128-1133.
39. http://www.healthcentral.com/prostate/psa-249759-5.html, *PSA Test Accuracy*, Accessed 05/05/2010
40. W. Gro Linno and V. Ben, *Acta Pathologica, Microbiologica et Immunologica Scandinavica* 2009, **118**, 85-90.
41. M. Zhou, A. M. Chinnaiyan, C. G. Kleer, P. C. Lucas and M. A. Rubin, *American Journal of Surgical Pathology*, 2002, **26**, 926-931.
42. F. Lin, R. E. Brown, T. Shen, X. J. Yang and C. Schuerch, *Applied Immunohistochemistry & Molecular Morphology*, 2004, **12**, 153-159.
43. O. Hameed, J. Sublett and P. A. Humphrey, *American Journal of Surgical Pathology*, 2005, **29**, 579-587.
44. M. Herawi, A. F. Parwani, J. Irie and J. I. Epstein, *American Journal of Surgical Pathology*, 2005, **29**, 874-880.
45. V. W. L. Ng, M. Koh, S. Y. Tan and P. H. Tan, *American Journal of Clinical Pathology*, 2007, **127**, 248-253.
46. P. Bydal, S. Auger and D. Poirier, *Steroids*, 2004, **69**, 325-342.
47. R. M. Brenner, O. D. Slayden, N. R. Nayak, D. T. Baird and H. O. D. Critchley, *Steroids*, 2003, **68**, 1033-1039.
48. G. Attard, A. H. M. Reid, T. A. Yap, F. Raynaud, M. Dowsett, S. Settatree, M. Barrett, C. Parker, V. Martins, E. Folkerd, J. Clark, C. S. Cooper, S. B. Kaye, D. Dearnaley, G. Lee and J. S. de Bono, *Journal of Clinical Oncology*, 2008, **26**, 4563-4571.
49. C. Ryan, E. Efstathiou, M. Smith, M. Taplin, G. Bubley, C. Logothetis, T. Kheoh, C. Haqq, A. Molina and E. J. Small, *J Clin Oncol (Meeting Abstracts)*, 2009, **27**, 5046-.
50. S. Ferdinandusse, H. Rusch, A. E. M. van Lint, G. Dacremont, R. J. A. Wanders and P. Vreken, *Journal of Lipid Research.*, 2002, **43**, 438-444.
51. J. Berg, T. Tymoczko. *Biochemistry, 6th* Edition, 2010.
52. http://www.blobs.org/science/article.php?article=32, Accessed 01/06/10, 2010.
53. R. J. A. Wanders, P. Vreken, S. Ferdinandusse, G. A. Jansen, H. R. Waterham, C. W. T. van Roermund and E. G. Van Grunsven, *Biochemical Society Transactions*, 2001, **29**, 250-267.
54. R. J. A. Wanders, S. Ferdinandusse, P. Brites and S. Kemp, *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, **1801**, 272-280.
55. M. Mukherji, C. J. Schofield, A. S. Wierzbicki, G. A. Jansen, R. J. A. Wanders and M. D. Lloyd, *Progress in Lipid Research*, 2003, **42**, 359-376.
56. N. M. Verhoeven, D. S. Roe, R. M. Kok, R. J. A. Wanders, C. Jakobs and C. R. Roe, *Journal of Lipid Research*, 1998, **39**, 66-74.

57. B. S. Jakobs and R. J. A. Wanders, *Biochemical and Biophysical Research Communications*, 1995, **213**, 1035-1041.
58. R. J. A. Wanders and H. R. Waterham, *Annual Review of Biochemistry*, 2006, **75**, 295-332.
59. M. C. Hunt and S. E. H. Alexson, *Progress in Lipid Research*, 2002, **41**, 99-130.
60. C. W. T. Vanroermund, Y. Elgersma, N. Singh, R. J. A. Wanders and H. F. Tabak, *European Molecular Biology Organisation*, 1995, **14**, 3480-3486.
61. http://themedicalbiochemistrypage.org/fatty-acid-oxidation.html, *Fatty acid oxidation*, Accessed 05/05/2010
62. P. Bhaumik, W. Schmitz, A. Hassinen, J. K. Hiltunen, E. Conzelmann and R. K. Wierenga, *Journal of Molecular Biology*, 2007, **367**, 1145-1161.
63. D. W. Russell, *Annual Review of Biochemistry*, 2003, **72**, 137-174.
64. G. F. Vanhove, P. P. Vanveldhoven, M. Fransen, S. Denis, H. J. Eyssen, R. J. A. Wanders and G. P. Mannaerts, *Journal of Biological Chemistry*, 1993, **268**, 10335-10344.
65. S. A. Filppula, R. T. Sormunen, A. Hartig, W. H. Kunau and J. K. Hiltunen, *Journal of Biological Chemistry*, 1995, **270**, 27453-27457.
66. S. Dalrymple, L. Antony, Y. Xu, A. R. Uzgare, J. T. Arnold, J. Savaugeot, L. J. Sokoll, A. M. De Marzo and J. T. Isaacs, *Cancer Research*, 2005, **65**, 9269-9279.
67. T. Kurosawa, M. Sato, T. Yoshimura, L. L. Jiang, T. Hashimoto and M. Tohma, *Biological & Pharmaceutical Bulletin*, 1997, **20**, 295-297.
68. D. A. Cuebas, C. Phillips, W. Schmitz, E. Conzelmann and D. K. Novikov, *Biochemical Journal*, 2002, **363**, 801-807.
69. U. Seedorf, P. Brysch, T. Engel, K. Schrage and G. Assmann, *Journal of Biological Chemistry*, 1994, **269**, 21277-21283.
70. http://www.medlink.com/medlinkcontent.asp, *Single enzyme defects of peroxisomal beta-oxidation*, Accessed 09/05/2010.
71. K. M. Knights, *Clinical And Experimental Pharmacology And Physiology*, 1998, **25**, 776-782.
72. J. N. Mubiru, G. L. Shen-Ong, A. J. Valente and D. A. Troyer, *Gene*, 2004, **327**, 89-98.
73. B. N. McLean, J. Allen, S. Ferdinandusse and R. J. A. Wanders, *Journal of Neurology, Neurosurgery & Psychiatry*, 2002, **72**, 396-399.
74. P. T. Clayton, *Biochemical Society Transactions*, 2001, **29**, 298-305.
75. K. Savolainen, T. J. Kotti, W. Schmitz, T. I. Savolainen, R. T. Sormunen, M. Ilves, S. J. Vainio, E. Conzelmann and J. K. Hiltunen, *Human Molecular Genetics*, 2004, **13**, 955-965.
76. M. D. Lloyd, D. J. Darley, A. S. Wierzbicki and M. D. Threadgill, *Federation of European Biochemical Societies*, 2008, **275**, 1089-1102.
77. J. N. Mubiru, A. J. Valente and D. A. Troyer, *Prostate*, 2005, **65**, 117-123.
78. H.-R. Li, J. Wang-Rodriguez, T. M. Nair, J. M. Yeakley, Y.-S. Kwon, M. Bibikova, C. Zheng, L. Zhou, K. Zhang, T. Downs, X.-D. Fu and J.-B. Fan, *Cancer Research*, 2006, **66**, 4079-4088.
79. S. Q. L. Zheng, B. L. Chang, D. A. Faith, J. R. Johnson, S. D. Isaacs, G. A. Hawkins, A. Turner, K. E. Wiley, E. R. Bleecker, P. C. Walsh, D. A. Meyers, W. B. Isaacs and J. F. Xu, *Cancer Research*, 2002, **62**, 6485-6488.
80. A. M. Levin, K. A. Zuhlke, A. M. Ray, K. A. Cooney and J. A. Douglas, *Prostate*, 2007, **67**, 1507-1513.
81. S. E. Daugherty, Y. Y. Shugart, E. A. Platz, M. D. Fallin, W. B. Isaacs, R. M. Pfeiffer, R. Welch, W. Y. Huang, D. Reding and R. B. Hayes, *Prostate*, 2007, **67**, 1487-1497.
82. R. S. Pereira, *Recent Patents on Anti-Cancer Drug Discovery*, 2009, **4**, 157-163.
83. F. R. Schmitz W, Conzelmann E, *European Journal of Biochemistry*, 1994, **222**, 313-323.
84. P. P. VanVeldhoven, K. Croes, M. Casteels and G. P. Mannaerts, *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism*, 1997, **1347**, 62-68.
85. N. M. Verhoeven and C. Jakobs, *Progress in Lipid Research*, 2001, **40**, 453-466.
86. F. A. Sattar, D. J. Darley, F. Politano, T. J. Woodman, M. D. Threadgill and M. D. Lloyd, *Chemical Communications*, **46**, 3348-3350.

87. P. Bhaumik, P. Kursula, V. Ratas, E. Conzelmann, J. K. Hiltunen, W. Schmitz and R. K. Wierenga, *Acta Crystallographica Section D-Biological Crystallography*, 2003, **59**, 353-355.
88. J. Heider, *Federation of European Biochemical Societies Letters*, 2001, **509**, 345-349.
89. W. B. White, C. V. Franklund, J. P. Coleman and P. B. Hylemon, *Journal of Bacteriology*, 1988, **170**, 4555-4561.
90. S. Ricagno, S. Jonsson, N. Richards and Y. Lindqvist, *European Molecular Biology Organisation*, 2003, **22**, 3210-3219.
91. S. Jonsson, S. Ricagno, Y. Lindqvist and N. G. J. Richards, *Journal of Biological Chemistry*, 2004, **279**, 36003-36012.
92. H. White and W. P. Jencks, *Journal of Biological Chemistry*, 1976, **251**, 1708-1711.
93. H. A. Barker, I. M. Jeng, N. Neff, J. M. Robertson, F. K. Tam and S. Hosaka, *Journal of Biological Chemistry*, 1978, **253**, 1219-1225.
94. F. Solomon and W. P. Jencks, *Journal of Biological Chemistry*, 1969, **244**, 1079-&.
95. T. Selmer and W. Buckel, *Journal of Biological Chemistry*, 1999, **274**, 20772-20778.
96. P. Dimroth and H. Eggerer, *Proceedings of the National Academy of Sciences of the United States of America*, 1975, **72**, 3458-3462.
97. S. Dickert, A. J. Pierik, D. Linder and W. Buckel, *European Journal of Biochemistry*, 2000, **267**, 3874-3884.
98. C. L. Berthold, C. G. Toyota, N. G. J. Richards and Y. Lindqvist, *Journal of Biological Chemistry*, 2008, **283**, 6519-6529.
99. K. Savolainen, P. Bhaumik, W. Schmitz, T. J. Kotti, E. Conzelmann, R. K. Wierenga and J. K. Hiltunen, *Journal of Biological Chemistry*, 2005, **280**, 12611-12620.
100. R. Brugger, C. Reichel, B. G. Alia, K. Brune, T. Yamamoto, I. Tegeder and G. Geisslinger, *Biochemical Pharmacology*, 2001, **62**, 399-399.
101. R. Mazumder, S. Ochoa, Y. Kaziro and T. Sasakawa, *Journal of Biological Chemistry*, 1962, **237**, 3065-&.
102. C. Reichel, R. Brugger, H. Bang, G. Geisslinger and K. Brune, *Molecular Pharmacology*, 1997, **51**, 576-582.
103. D. J. Darley, D. S. Butler, S. J. Prideaux, T. W. Thornton, A. D. Wilson, T. J. Woodman, M. D. Threadgill and M. D. Lloyd, *Organic & Biomolecular Chemistry*, 2009, **7**, 543-552.
104. V. M. Powers, C. W. Koo, G. L. Kenyon, J. A. Gerlt and J. W. Kozarich, *Biochemistry*, 1991, **30**, 9255-9263.
105. P. F. Leadlay and J. Q. Fuller, *Biochemical Journal*, 1983, **213**, 635-642.
106. J. S. Wiseman and J. S. Nichols, *Journal of Biological Chemistry*, 1984, **259**, 8907-8914.
107. V. M. Powers, C. W. Koo, G. L. Kenyon, J. A. Gerlt and J. W. Kozarich, *Biochemistry*, 1991, **30**, 9255-9263.
108. S. Glavas and M. E. Tanner, *Biochemistry*, 1999, **38**, 4106-4113.
109. B. Antonella, M. Toshiro, T. Dan and L. Massimo, *Journal of Cellular Biochemistry*, 2004, **91**, 47-53.
110. I. Jonathan, C. Marna and W. Alan, *Urology*, 1995, **45**, 81-86.
111. S. Rossi, E. Graner, P. Febbo, L. Weinstein, N. Bhattacharya, T. Onody, G. Bubley, S. Balk and M. Loda, *Molecular Cancer Research*, 2003, **1**, 707-715.
112. T. M. Loftus, D. E. Jaworsky, G. L. Frehywot, C. A. Townsend, G. V. Ronnett, M. D. Lane and F. P. Kuhajda, *Science*, 2000, **288**, 2379-2381.
113. S. Zha, S. Ferdinandusse, J. L. Hicks, S. Denis, T. A. Dunn, R. J. Wanders, J. Luo, A. M. De Marzo and W. B. Isaacs, *Prostate*, 2005, **63**, 316-323.
114. Z. Shan, F. Sacha, L. H. Jessica, D. Simone, A. D. Thomas, J. W. Ronald, L. Jun, M. D. M. Angelo and B. I. William, *The Prostate*, 2005, **63**, 316-323.
115. A. M. De Marzo, A. K. Meeker, S. Zha, J. Luo, M. Nakayama, E. A. Platz, W. B. Isaacs and W. G. Nelson, *Urology*, 2003, **62**, 55-62.

116. K. Takahara, H. Azuma, T. Sakamoto, S. Kiyama, T. Inamoto, N. Ibuki, T. Nishida, H. Nomi, T. Ubai, N. Segawa and Y. Katsuoka, *Anticancer Research*, 2009, **29**, 2497-2505.
117. A. J. Carnell, I. Hale, S. Denis, R. J. A. Wanders, W. B. Isaacs, B. A. Wilson and S. Ferdinandusse, *Journal of Medicinal Chemistry*, 2007, **50**, 2700-2707.
118. V. Copie, W. S. Faraci, C. T. Walsh and R. G. Griffin, *Biochemistry*, 1988, **27**, 4966-4970.
119. W. S. Faraci and C. T. Walsh, *Biochemistry*, 1989, **28**, 431-437.
120. W. Seubert and F. Lynen, *Journal of the American Chemical Society.*, 1953, **75**, 2787-2788.
121. L. R. a. B. Kass, D. J. H, *Methods in Enzymology*, 1969, **14**, 696-698.
122. C. Lever, X. Li, R. Cosstick, S. Ebel and T. Brown, *Nucleic Acids Research*, 1993, **21**, 1743-1746.
123. M. Kuzuya, N. Yokota, A. Noguchi and T. Okuda, *Bulletin of the Chemical Society of Japan*, 1986, **59**, 1379-1385.
124. P. Arora, A. Vats, P. Saxena, D. Mohanty and R. S. Gokhale, *Journal of the American Chemical Society.*, 2005, **127**, 9388-9389.
125. P. Hoyos, M. Hernandez, J. V. Sinisterra and A. R. Alcantara, *Journal of Organic Chemistry*, 2006, **71**, 7632-7637.
126. I. Hale, Thesis, University of Liverpool, 2006.
127. B. Dolensky, J. Kvicala, J. Palecek and O. Paleta, *Journal of Fluorine Chemistry*, 2002, **115**, 67-74.
128. J. Sugimoto, K. Miura, K. Oshima and K. Utimoto, *Chemistry Letters*, 1991, **20**, 1319-1322.
129. S. Watanabe, Y. Shimada, T. Kitazume and T. Yamazaki, *Journal of Fluorine Chemistry*, 1992, **59**, 249-256.
130. P. D. G. Dean, *Journal of the Chemical Society*, 1965, 6655-&.
131. N. G. Clayden J, Stuart Warren, Peter Wothers *Organic Chemistry*, 2001.
132. H. A. J. Furniss B, Smith P.W.G, Tatchell A.R, *Vogal-Organic Synthesis*, 1991.
133. K. Parang, E. E. Knaus, L. I. Wiebe, S. Sardari, M. Daneshtalab and F. Csizmadia, *Archiv der Pharmazie*, 1996, **329**, 475-482.
134. B. K. Park, N. R. Kitteringham and P. M. O'Neill, *Annual Review of Pharmacology and Toxicology*, 2001, **41**, 443-470.
135. S. J. Blanksby and G. B. Ellison, *Accounts of Chemical Research*, 2003, **36**, 255-263.
136. R. W. Winter, J. X. Kelly, M. J. Smilkstein, R. Dodean, G. C. Bagby, R. K. Rathbun, J. I. Levin, D. Hinrichs and M. K. Riscoe, *Experimental Parasitology*, 2006, **114**, 47-56.
137. G. S. Lal, G. P. Pez, R. J. Pesaresi, F. M. Prozonic and H. Cheng, *Journal of Organic Chemistry*, 1999, **64**, 7048-7054.
138. *US patent 20060194801 Pat.*, 20060194801, 2006.
139. S. Wang, W. Mao, Z. She, C. Li, D. Yang, Y. Lin and L. Fu, *Bioorganic & Medicinal Chemistry Letters*, 2007, **17**, 2785-2788.
140. C. D. Leigh and C. R. Bertozzi, *Journal of Organic Chemistry*, 2008, **73**, 1008-1017.
141. K. Tomioka, A. Muraoka and M. Kanai, *Journal of Organic Chemistry*, 1995, **60**, 6188-6190.
142. M. H. Bhure, C. V. Rode, R. C. Chikate, N. Patwardhan and S. Patil, *Catalysis Communications*, 2007, **8**, 139-144.
143. W. J. Middleton and E. M. Bingham, *Journal of Fluorine Chemistry*, 1983, **22**, 561-574.
144. Y. Yamauchi, S. Hara and H. Senboku, *Tetrahedron*, **66**, 473-479.
145. W. R. Shieh, D. M. Gou, Y. C. Liu, C. S. Chen and C. Y. Chen, *Analytical Biochemistry*, 1993, **212**, 143-149.
146. J. C. Vincent, W. H. Gordon and L. T. Donald, *Journal of Labelled Compounds and Radiopharmaceuticals*, 1986, **23**, 187-196.
147. R. E. Gawley, *Tetrahedron Letters*, 1999, **40**, 4297-4300.
148. V. M. Kanagasabapathy, J. F. Sawyer and T. T. Tidwell, *Journal of Organic Chemistry*, 1985, **50**, 503-509.
149. *US patent 4952718 Pat.*, 4952718 1990.
150. T. Ozturk, E. Ertas and O. Mert, *Chemical Reviews*, **110**, 3419-3478.

151. B. Jiang and Y. Xu, *Journal of Organic Chemistry*, 1991, **56**, 7336-7340.
152. L. Acemoglu and J. M. J. Williams, *Journal of Molecular Catalysis A: Chemical*, 2003, **196**, 3-11.
153. E. Ishiguro S. Iwata, Y. Suzuki, A Mikuni and T Sasaki., *Journal of Physics B: Atomic and Molecular Physics*, 1982, **15**, 1841.
154. P. R. Rablen, *J. Am. Chem. Soc.*, 1997, **119**, 8350-8360.
155. K. Kato, Y.-f. Gong, S. Tanaka, M. Katayama and H. Kimoto, *Biotechnology Letters*, 1999, **21**, 457-461.
156. M. T. F. Ernest L. Eliel, and Thomas Prosser, *Organic Syntheses*, 1963, **4**.
157. L. Haughton and J. M. J. Williams, *Synthesis*, 2001, **2001**, 0943-0946.
158. M. Smith, J. March, *March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure* Wiley-Blackwell; 6th Edition edition 2007.
159. F. Mathew and B. Myrboh, *Tetrahedron Letters*, 1990, **31**, 3757-3758.
160. H. L. van Maanen, H. Kleijn, J. T. B. H. Jastrzebski, M. T. Lakin, A. L. Spek and G. van Koten, *Journal of Organic Chemistry*, 1994, **59**, 7839-7848.
161. M. A. Fredrick and M. Hulce, *Tetrahedron*, 1997, **53**, 10197-10227.
162. M. Bryhn, A. Holmeide and K Kristin, *US patent*, 2006117664.
163. H. Fujisawa, T. Fujiwara, Y. Takeuchi and K. Omata, *Chemical & Pharmaceutical Bulletin*, 2005, **53**, 524-528.
164. U. Sidenius, C. Skonberg, J. Olsen and S. H. Hansen, *Chemical Research in Toxicology*, 2004, **17**, 75-81.
165. J. E. Moses, J. E. Baldwin, S. Bruckner, S. J. Eade and R. M. Adlington, *Organic & Biomolecular Chemistry*, 2003, **1**, 3670-3684.
166. A. F. Littke and G. C. Fu, *Journal of the American Chemical Society.*, 2001, **123**, 6989-7000.
167. A. Whitty, C. A. Fierke and W. P. Jencks, *Biochemistry*, 1995, **34**, 11678-11689.
168. C. J. Arthur, A. E. Szafranska, J. Long, J. Mills, R. J. Cox, S. C. Findlow, T. J. Simpson, M. P. Crump and J. Crosby, *Chemistry & Biology*, 2006, **13**, 587-596.
169. P. Arora, A. Vats, P. Saxena, D. Mohanty and R. S. Gokhale, *Journal of the American Chemical Society.*, 2005, **127**, 9388-9389.
170. Z. T. Pei, N. A. Oey, M. M. Zuidervaart, Z. Z. Jia, Y. Y. Li, S. J. Steinberg, K. D. Smith and P. A. Watkins, *Journal of Biological Chemistry.*, 2003, **278**, 47070-47078.
171. A. S. Kende and B. H. Toder, *Journal of Organic Chemistry*, 1982, **47**, 163-167.
172. P. V. Schleyer, J. D. Dill, J. A. Pople and W. J. Hehre, *Tetrahedron*, 1977, **33**, 2497-2501.
173. T. S. Tracy and S. D. Hall, *Drug Metabolism and Disposition*, 1992, **20**, 322-327.
174. D. Ouazia and S. L. Bearne, *Analytical Biochemistry*, 2010, **398**, 45-51.
175. S. L. Asa, K. Somers and S. Ezzat, *Journal of Clinical Endocrinology & Metabolism*, 1998, **83**, 3210-3212.
176. R. D. Barber, D. W. Harmer, R. A. Coleman and B. J. Clark, *Physiological Genomics*, 2005, **21**, 389-395.
177. L. Ute, K.-K. Angelika, B. Angelika and E. Hermann, *European Journal of Biochemistry*, 1991, **198**, 767-773.
178. J. Hasegawa, M. Hamada, T. Miyamoto, K. Nishide, T. Kajimoto, J. Uenishi and M. Node, *Carbohydrate. Research.*, 2005, **340**, 2360-2368.
179. M. Ohkubo, S. Mochizuki, T. Sano, Y. Kawaguchi and S. Okamoto, *Organic Letters*, 2007, **9**, 773-776.
180. C. Davide, F. Kevin, S. Ania, G. Veronique and R. Marina, *Chemistry - A European Journal*, 2008, **14**, 7059-7065.
181. B. S. Bodnar and P. F. Vogt, *Journal of Organic Chemistry*, 2009, **74**, 2598-2600.
182. F. Bellezza, A. Cipiciani, G. Ricci and R. Ruzziconi, *Tetrahedron*, 2005, **61**, 8005-8012.
183. O. Goj, S. Kotila and G. Haufe, *Tetrahedron*, 1996, **52**, 12761-12774.

184. M. Moghadam, S. Tangestaninejad, V. Mirkhani, I. Mohammadpoor-baltork, N. Sirjanian and S. Parand, *Bioorganic & Medicinal Chemistry*, 2009, **17**, 3394-3398.
185. A. Gonzalez, *Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry*, 1991, **21**, 1353-1360.
186. S. C. Gregory, D. Marco, E. Jason, F. Anthony and N. Julian, *Journal of Labelled Compounds and Radiopharmaceuticals*, 2006, **49**, 903-914.
187. X. Cheng, Q. Zhang, J. H. Xie, L. X. Wang and Q. L. Zhou, *Angewandte Chemie International Edition*, 2005, **44**, 1118-1121.
188. Y. Liu, D. Mao, D. Xu, Z. Xu and Y. Zhang, *Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry*, 2007, **37**, 4389 - 4397.
189. F. Bargiggia and O. Piva, *Tetrahedron: Asymmetry*, 2003, **14**, 1819-1827.